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(71) Applicant (for all designated States except US): **UNIVERSITÄT ZÜRICH [CH/CH]**; Rämistrasse 71, CH-8006 Zürich (CH).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **ALBERTO, Roger [CH/CH]**; St. Georgenstrasse 54, CH-8400 Winterthur (CH). **PAK, Jaw, Kyoung [KR/CH]**; Raingaessli, CH-6340 Baar-Inwil (CH).

(74) Agent: **VAN SOMEREN, Petronella, Francisca, Hendrika, Maria**; Arnold & Siedsma, Sweelinckplein 1, NL-2517 GK The Hague (NL).

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(54) Title: **N<sup>ε</sup> AND/OR N<sup>α</sup> DERIVATIZED, METAL AND ORGANIC PROTECTED L-HISTIDINE FOR COUPLING TO BIOMOLECULES FOR HIGHLY EFFICIENT LABELING WITH [M(OH)<sub>2</sub>(CO)<sub>3</sub>]<sup>+</sup> BY FAC COORDINATION**

(57) Abstract: The present invention relates to novel histidine derivatives that can be used for the labeling of biomolecules with radioactive metal tricarbonyls. The new derivatives have a histidine that is derivatized at the N<sup>ε</sup> and at least protected at the N<sup>α</sup> and optionally at the N<sup>δ</sup>; or derivatized at the N<sup>α</sup> and at least protected at the N<sup>α</sup> and optionally at the N<sup>δ</sup>; or derivatized at the N<sup>ε</sup> and N<sup>α</sup> and at least protected at the N<sup>α</sup> and optionally at the N<sup>δ</sup>; or derivatized at the N<sup>ε</sup>; or derivatized at the N<sup>α</sup>; or derivatized at the N<sup>ε</sup> and N<sup>α</sup>; or at least protected at the N<sup>α</sup> and optionally at the N<sup>δ</sup>.

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N<sup>c</sup> AND/OR N<sup>a</sup> DERIVATIZED, METAL AND ORGANIC PROTECTED  
L-HISTIDINE FOR COUPLING TO BIOMOLECULES FOR HIGHLY EFFICIENT  
LABELING WITH  $[M(OH_2)_3(CO)_3]^+$  BY *fac* COORDINATION

5           The invention relates to new histidine derivatives that can be coupled to biomolecules, such as amino acids, peptides etc. for labeling with a radioactive metal tricarbonyl  $[M(OH_2)_3(CO)_3]^+$  by *fac* coordination.

          The labeling of biologically active molecules with  
10  $^{99m}Tc$  for radiopharmaceutical purposes is a field of intense research. The commercially available perfusion agents for radioimaging have to be complemented by labeled vectors which will allow a more precise targeting of various receptors expressed in higher density on i.e. cancer cells. So far, a  
15 few compounds are in pre-clinical trials but none has found commercial application so far.

          Many chemical and biological difficulties have to be overcome. Chemically, the targeting vector has to be i) derivatized with an appropriate chelator for  $^{99m}Tc$  or other  
20 radionuclide, ii) should be labeled at high specific activity (low vector concentration) and finally retain its physico-chemical properties and its affinity towards the corresponding receptor. For routine use, the labeling process must be performed preferentially in one single step.

25           Different procedures are available from literature and for peptides in particular the hynic approach seems to be promising although it suffers from the lack of a clearly defined compound which is required for clinical approval.

          The present inventors recently presented the one pot  
30 synthesis of the organometallic aqua-ion  $[^{99m}Tc(OH_2)_3(CO)_3]^+$  (Alberto et al., J. Am. Chem. Soc. 2001, 123, 3135) and

showed the versatility of using this complex fragment for the labeling of various biomolecules and peptides in particular.

One of the major advantages of the carbonyl approach is the availability of a well-defined complex with very high specific activity only depending from the ligand type.  
5 Naturally occurring bidentate ligands such as N-terminal histidines in peptide chains can efficiently be labeled with  $[^{99m}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ . An improvement in respect of specific activity was the introduction of a terminal histidine through  
10 the  $\alpha$ -amino group which allowed labeling at low ligand concentration.

However, this type of bifunctional chelator has a relatively high lipophilicity and its synthesis is difficult.

It is therefore the object of the invention to  
15 provide a differently derivatized histidine which would allow its introduction into or onto any peptide with a minimum of synthetic work and a maximum of labeling efficiency.

The inventors contemplated that since the complex  $[^{99m}\text{Tc}(\text{his})(\text{CO})_3]$  is hydrophilic it is appropriate to  
20 derivatize the histidine at the  $\epsilon$ -nitrogen in the imidazole ring. This functionalization leaves the highly efficient tripodal coordination site untouched but still allows the coupling to amine or carboxylic groups in biomolecules. Finally, since the synthesis of  $[^{99m}\text{Tc}(\text{his})(\text{CO})_3]$  can be  
25 performed in one single step from  $[^{99m}\text{TcO}_4]^-$ , histidine also fulfils the requirement for a one pot labeling procedure without affecting the ligand.

The invention thus relates to histidine derivatives, comprising a histidine that is any one of the following:

30 a) derivatized at the  $\text{N}^\epsilon$  and at least protected at the  $\text{N}^\alpha$  and optionally at the  $\text{N}^\delta$ ; or

b) derivatized at the  $N^a$  and at least protected at the  $N^a$  and optionally at the  $N^b$ ; or

c) derivatized at the  $N^c$  and  $N^a$  and at least protected at the  $N^a$  and optionally at the  $N^b$ ; or

5 d) derivatized at the  $N^c$ ; or

e) derivatized at the  $N^a$ ; or

f) derivatized at the  $N^c$  and  $N^a$ ; or

g) at least protected at the  $N^a$  and optionally at the  $N^b$ .

10 The  $N^c$  and/or  $N^a$  are derivatized with  $-(CH_2)_n-R$  wherein  $n = 0, 1, 2, 3, 4, 5, 6, 7, 8, 9$  or  $10$ , preferably  $1, 2, 3, 4$  or  $5$ , and  $R$  is a group selected from  $-NH_2$ ,  $-COOR_1$ ,  $-OH$ ,  $-X$  or  $-X'$ -biomolecule, wherein  $X'$  is a coupling block having a bond that is the result from a reaction between  $COOH$   
15 and  $NH_2$ ,  $NH_2$  and  $COOH$ ,  $OH$  and  $Ph-OH$ , wherein  $Ph$  is phosphoric acid group on the biomolecules, such as phosphorylated peptide or glycosyl phosphates or  $X$  and an electrophilic functional group on the biomolecule, in particular  $S$ ,  $OH$  or amine and  $R_1$  is  $H$ ,  $t$ -butyl or pentafluorophenyl.  $X$  is  
20 suitably selected from halides, azides, pseudohalides, phosphate, thiol, silyl.

Either or both of  $N^c$  and  $N^a$  can be derivatized with a biomolecule. This can be any biomolecule, in particular polypeptides, such as antibodies, peptides, amino acids,  
25 sugars, vitamins. Suitable examples of biomolecules are bombesine, (alpha)-MSH peptides, such as melanocortin, octreotate, somatostatin, interleukin-8 (IL8), CCK, (beta)-hairpin loop peptides, neurotensin, biotin, monoclonal antibodies, such as monoclonal antibodies directed against  
30 prostate membrane specific antigen (pmsa).

The biomolecule can be coupled directly to the N<sup>ε</sup> and/or N<sup>α</sup> or the N<sup>ε</sup> and/or N<sup>α</sup> can be first derivatized with a group of the formula  $-(CH_2)_n-R$ , wherein R is as defined above.

- 5           The N<sup>α</sup> and N<sup>δ</sup> can be protected with a carbonyl thus forming a six-membered urea ring. Alternatively, N<sup>α</sup>, N<sup>δ</sup> and the carboxyl group are protected with a metal tricarbonyl.

These two forms of protection are in particular useful when derivatization with  $-(CH_2)_n-R$  is to take place.

10 When subsequently this group is further derivatized with a biomolecule the original protection may be replaced by protection of N<sup>α</sup> with a amine protecting group, in particular Fmoc, Cbz, BOC, Teoc, methoxycarbonyl, ethoxycarbonyl, and protection of the carboxyl group by esterification.

- 15           After all derivatization steps are completed the histidine derivative can be deprotected and subsequently coordinated to a radioactively labeled metal tricarbonyl to obtain a labeled biomolecule.

The radioactively labeled metal tricarbonyl is

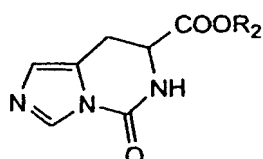
20 suitably selected from  $[^{99m}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ ,  $[^{188}\text{Re}(\text{OH}_2)_3(\text{CO})_3]^+$  and  $[^{97}\text{Ru}(\text{OH}_2)_3(\text{CO})_3]^{2+}$ .

According to a further aspect of the invention it was found that coupling of the biomolecule is highly facilitated when the  $-(CH_2)_n-R$  on N<sup>ε</sup> is derivatized as  $-(CH_2)_n-\text{COO}-$

25 pentafluorophenyl ester. This derivatization leads to an activation of the COOH<sup>-</sup> on N<sup>ε</sup>, which gives a possibility of direct conjugation with biomolecules without any modification when a biomolecules itself has free carboxylic acid that might be competitive for coupling. In the above situation N<sup>α</sup>

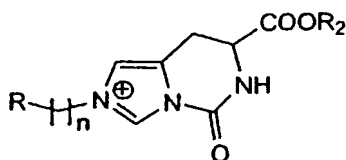
30 and the carboxylic part are protected.

The present invention relates in particular to histidine derivatives having one of the following structural formulas



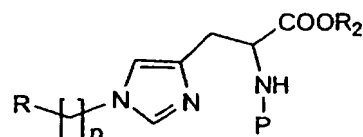
I

$R_2 = \text{H, methyl}$



II

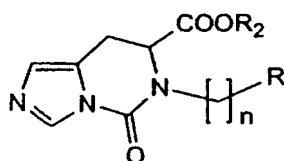
$R = \text{NH}_2, \text{COOR}_1, \text{OH, X, X'-biomolecule}$   
 $R_1 = \text{H, } t\text{-butyl}$   
 $R_2 = \text{H, methyl}$



III

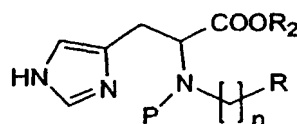
$R = \text{NH}_2, \text{COOR}_1, \text{OH, X, X'-biomolecule}$   
 $R_1 = \text{H, } t\text{-butyl, Pfp}$   
 $R_2 = \text{H, methyl}$   
 $P = \text{H, Fmoc, Cbz, BOC, Teoc, methoxycarbonyl, ethoxycarbonyl}$

5



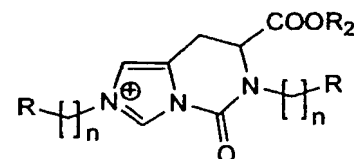
IV

$R = \text{H, COOR}_1$   
 $R_1 = \text{H, } t\text{-butyl}$   
 $R_2 = \text{H, methyl}$



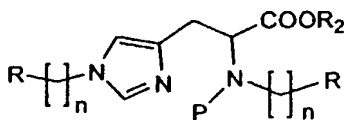
V

$R = \text{H, COOR}_1$   
 $R_1 = \text{H, } t\text{-butyl}$   
 $R_2 = \text{H, methyl}$   
 $P = \text{H, Fmoc}$



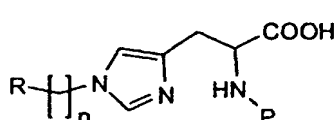
VI

$R = \text{NH}_2, \text{COOR}_1, \text{OH, X}$   
 $R_1 = \text{H, } t\text{-butyl}$   
 $R_2 = \text{H, methyl}$



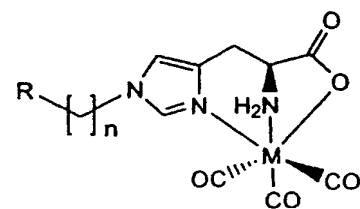
VII

$R = \text{H, COOR}_1$   
 $R_1 = \text{H, } t\text{-butyl}$   
 $R_2 = \text{H, methyl}$   
 $P = \text{H, Fmoc}$



VIII

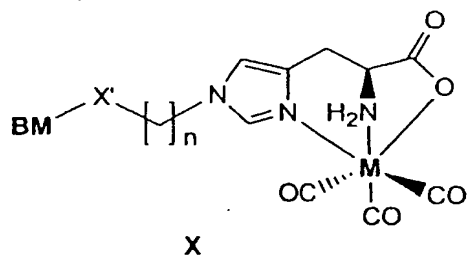
$R = \text{H, COOR}_1$   
 $R_1 = \text{H, } t\text{-butyl}$   
 $P = \text{H, Fmoc}$



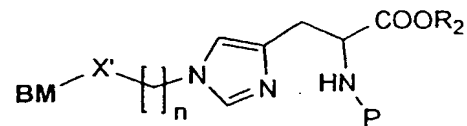
IX

$M = \text{Re, } ^{99m}\text{Tc, Ru}$   
 $R = \text{NH}_2, \text{COOH, OH, X}$

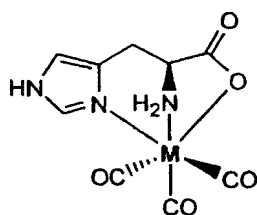
6



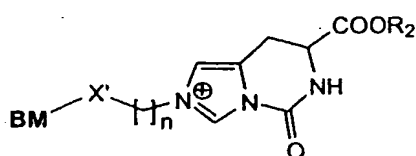
M = Re, <sup>99m</sup>Tc, Ru  
BM = biomolecule



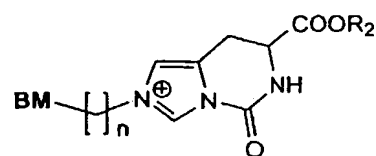
R<sub>2</sub> = H, methyl  
P = H, Fmoc, Cbz, Teoc



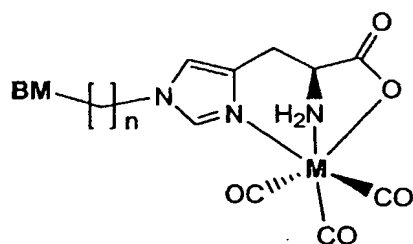
M = Re, <sup>99m</sup>Tc



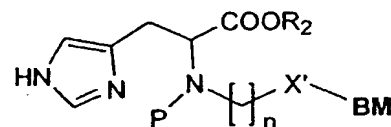
R<sub>2</sub> = H, methyl  
BM = biomolecule



R<sub>2</sub> = H, methyl  
BM = biomolecule

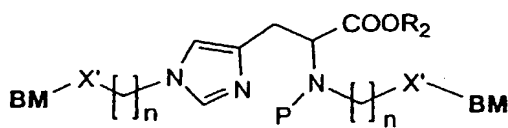


M = Re, <sup>99m</sup>Tc  
BM = biomolecule

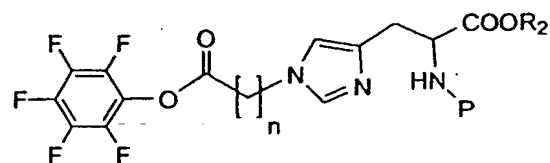


R<sub>2</sub> = H, methyl  
P = H, Fmoc  
BM = biomolecule

5



R<sub>2</sub> = H, methyl  
P = H, Fmoc  
BM = biomolecule



R<sub>2</sub> = H, methyl  
P = H, Fmoc

According to a further aspect thereof, the invention relates to biomolecules coupled to a histidine derivative as claimed. The histidine can be at the end or within the biomolecule. Alternatively, both N<sup>ε</sup> and N<sup>α</sup> can be derivatized with a biomolecule leading to dimers or to bifunctional molecules. Examples of bifunctional molecules are molecules in which one side of the biomolecule has a targeting function, such as an antibody or a ligand to a receptor and the other side of biomolecule is used for its toxicity. Other combinations are also part of this invention. Such bifunctional molecules can for example be used for the targeted treatment of tumors. The targeting to a tumor will bring the toxic biomolecule and the radioactive metal in the vicinity of the tumor to be treated.

Suitable biomolecules are bombesine, (alpha)-MSH peptides, such as melanocortin, octreotate, somatostatin, interleukin-8 (IL8), CCK, (beta)-hairpin loop peptides, neurotensin, biotin, monoclonal antibodies, such as monoclonal antibodies directed against prostate membrane specific antigen (pmsa).

In the research that led to the invention two different pathways were found for the introduction of an acetyl group at N<sup>ε</sup> in N<sup>α</sup>, N<sup>δ</sup> and -COO protected histidine to afford the model compound N<sup>ε</sup>-(CH<sub>2</sub>COOH)-histidine derivative 9. Compounds of the invention, like histidine derivative 9, can be coupled to amino groups in bioactive molecules such as peptides. After full deprotection of such a bioconjugate, histidine provides three coordination sites which efficiently coordinate to [<sup>99m</sup>Tc(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>+</sup> or [Re(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>+</sup> [<sup>97</sup>Ru(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>2+</sup> in a facial geometry.

The invention thus also provides a method for preparing histidine derivatives of the invention, comprising:



- a) providing histidine;  
b) protecting at least the  $N^{\alpha}$  and optionally the carboxyl and the  $N^{\delta}$ ;  
c) derivatizing at least one of the  $N^{\epsilon}$  and  $N^{\alpha}$ ; and  
5 d) deprotecting the protected groups.

The method may further comprise the step e) of labeling the deprotected compound to obtain a labeled compound.

In the method the  $N^{\alpha}$  and  $N^{\delta}$  may be protected by a  
10 carbonyl group thus forming a six-membered urea ring or the carboxyl,  $N^{\alpha}$  and  $N^{\delta}$  may be coordinated to a metal, in particular a metal tricarbonyl.

The derivatization of  $N^{\epsilon}$  and/or  $N^{\alpha}$  can be performed with  $-(CH_2)_n-R$  wherein  $n = 0, 1, 2, 3, 4, 5, 6, 7, 8, 9$  or  
15 10, preferably 1, 2, 3, 4 or 5, and R is a group selected from  $-NH_2$ ,  $-COOR_1$ ,  $-OH$ ,  $-X$  or  $-X'$ -biomolecule, wherein  $X'$  is a coupling block having a bond that is the result from a reaction between  $COOH$  and  $NH_2$ ,  $NH_2$  and  $COOH$ ,  $OH$  and  $Ph-OH$ , wherein Ph is phosphoric acid group on the biomolecules, such  
20 as phosphorylated peptide or glycosyl phosphates or X and an electrophilic functional group on the biomolecule, in particular S, OH or amine, wherein  $R_1$  is H, t-butyl.

Alternatively,  $N^{\epsilon}$  and/or  $N^{\alpha}$  can be directly derivatized with a biomolecule.

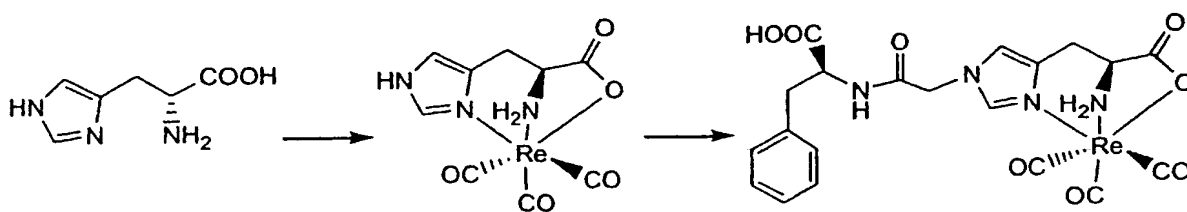
25 When protection is achieved by means of the urea ring and derivatization of  $-(CH_2)_n-R$  takes place at  $N^{\alpha}$ , the ring may be opened prior to introducing the biomolecule. In that case the carboxyl is protected by esterification and the  $N^{\alpha}$  is protected with an amine protecting group, such as Fmoc,  
30 Cbz, BOC, Teoc, methyloxycarbonyl, or ethyloxycarbonyl group.

In order to facilitate derivatization,  $-(CH_2)_n-R$  on  $N^{\epsilon}$  may first be derivatized as  $-(CH_2)_n-COO$ -pentafluorophenyl ester.

When the method comprises the step of labeling the  
 5 derivative this is suitably done with a radioactively labeled metal tricarbonyl, in particular a radioactively labeled metal tricarbonyl selected from  $[^{99m}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ ,  $[^{188}\text{Re}(\text{OH}_2)_3(\text{CO})_3]^+$  and  $[^{97}\text{Ru}(\text{OH}_2)_3(\text{CO})_3]^{2+}$ .

Selective derivatization at the  $N^{\epsilon}$  position has thus  
 10 conveniently been achieved by concomitant protection of  $N^{\alpha}$  and  $N^{\delta}$  with a carbonyl group forming a six-membered urea. Cyclic urea ring opening with  $\text{Fm-OH}$ , coupling of phenyl-alanine as a model to **9** through its primary amine and removal of all protecting groups in one step gave a histidine  
 15 derivative of phenyl-alanine which could be labeled at  $10^{-5}$  M with  $^{99m}\text{Tc}$  in very high yield and even in about 50% yield at  $10^{-6}$  M. The x-ray structure of a complex with  $[\text{Re}(\text{CO})_3]^+$  in which anilin is coupled to **9** confirms the facial arrangement of histidine.

20 A second pathway applies directly the  $[\text{Re}(\text{CO})_3]^+$  moiety as a protecting group as shown in the scheme below.



25

This is one of the rare examples in which a metal fragment is used as a protecting group for organic functionalities.

The coordination to histidine protects  $N^{\alpha}$ ,  $N^{\delta}$  and  $-\text{COO}$  in one single step, subsequent alkylation with  $\text{BrCH}_2\text{COOH}(\text{R})$  at  $N^{\epsilon}$ , coupling to phenyl-alanine and oxidative deprotection of  $[\text{Re}(\text{CO})_3]^+$  to  $[\text{ReO}_4]^-$  gave the corresponding  
5 bioconjugate in which histidine is coupled to phenyl-alanine through an acetylamide at  $N^{\epsilon}$ .

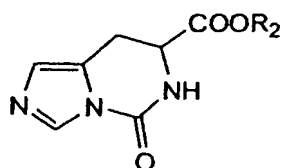
Both methods offer convenient pathways to introduce histidine in a biomolecule under retention of its three coordination sites. The procedures are adaptable to any  
10 biomolecule with pendant amines and allow the development of novel radiopharmaceuticals or inversed peptides.

Thus, a high yield labeling of biomolecules with  $[\text{}^{99\text{m}}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$  is possible at  $\mu\text{M}$  concentrations, when histidine is linked through  $N^{\epsilon}$  in the imidazole ring to a  
15 targeting molecule. Two convenient strategies to produce such derivatives have been worked out, one employing the  $[\text{Re}(\text{CO})_3]^+$  core as an organometallic protecting group for three functionalities in histidine. The key compounds can be coupled to any amino group in a biomolecule and be  
20 labeled in one single step from  $[\text{}^{99\text{m}}\text{TcO}_4]^-$  in water which enables the development of new radiopharmaceuticals.

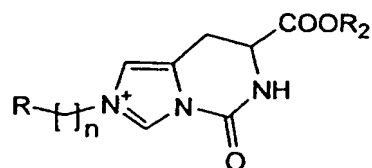
The derivatization method of choice depends on the biomolecule to be coupled. The opening of the urea ring requires acidic pH and reducing conditions but is generally  
25 performed prior to biomolecule coupling. This method is suitable for biomolecules that can withstand such conditions, such as vitamins. Alternatively, polypeptides can be coupled to a histidine that is protected with the metal carbonyl.

Below examples are listed of various types of  
30 histidine derivatives for highly efficient and biologically stable labeling of biomolecules.

$N^{\alpha}$  and  $N^{\delta}$  can be protected in one single step, leaving  $N^{\epsilon}$  free for further derivatizations. Derivatizations at  $N^{\alpha}$  are possible as well.



$R_2 = \text{H, methyl}$



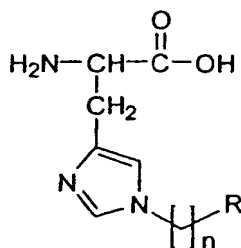
$R = \text{NH}_2, \text{COOR}_1, \text{OH, X}$

$R_1 = \text{H, } t\text{-butyl}$

$R_2 = \text{H, methyl}$

(Molecule 1)

The left molecule shows the histidine in which  $N^{\alpha}$  and  $N^{\delta}$  are protected and  $N^{\epsilon}$  is left for derivatization, which is shown in the right hand molecule. Derivatization (such as alkylation) at  $N^{\epsilon}$  leads to a variety of derivatives that can be coupled to biomolecules through the pendant functionality, which can be an amine, a carboxylate, a halide and others. This kind of synthesis is essentially literature known (R. Jain, et al, J. Chem. Soc., Dalton Trans, 1994, 2815). After deprotection, a histidine derivative with possibility of tripodal coordination via the  $N^{\alpha}$ , to  $[\text{M}(\text{CO})_3]^+$  remains:



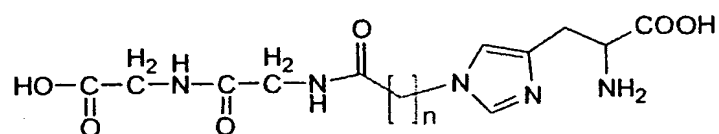
$R = \text{NH}_2, \text{COOR}_1, \text{OH, X}$

$R_1 = \text{H, Pentafluorophenol}$

Before deprotection, molecule 1 can be coupled to a biomolecule of any kind. R can thus also be a biomolecule.

Deprotection yields then again a biomolecule that contains a tripodal histidine ligand. This ligand is of highest efficiency in terms of labeling with  $^{99m}\text{Tc}$  or  $^{188}\text{Re}$  and allows the labeling of biomolecules almost on the n.c.a. level.

5 The following is an example of tripodal histidine coupled to a model peptide sequence:

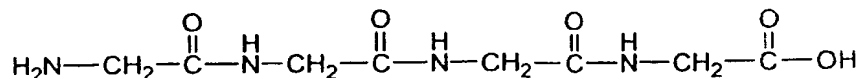


tripodal histidine

10

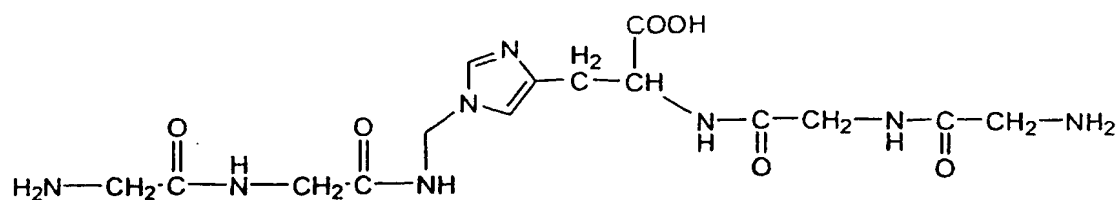
The combination of synthetic techniques and biomolecule labeling is novel and the high yields unexpected. Moreover, according to the invention the very powerful carbonyl ligand can now be coupled to a biomolecule very easily. Derivatives of the above mentioned type can be coupled to essentially any biomolecule under retention of its physico-chemical properties. Histidine coupled in the way according to the invention is a natural ligand.

Furthermore, histidine derivatives of the invention can be used to reverse the direction of a peptide chain. A normal peptide sequence has for example the following structural formula:

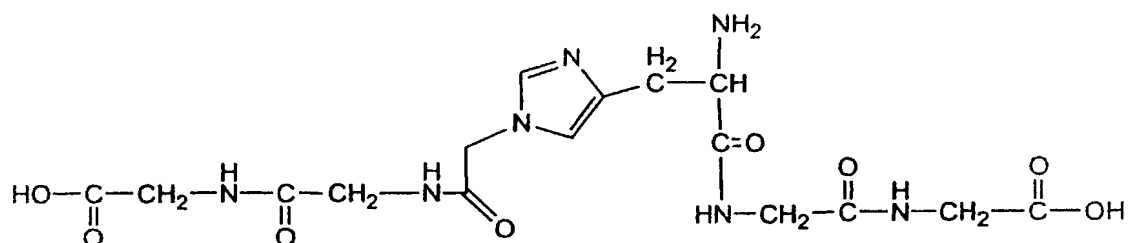


25

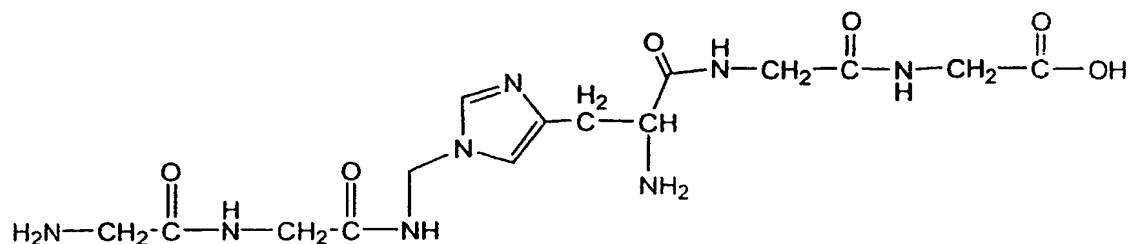
By means of the histidine derivatives of the invention reversed sequences can be produced to yield two N-termini and a bidentate his ligand:



Alternatively a reversed sequence with two C-termini  
5 and a bidentate his ligand can be obtained:

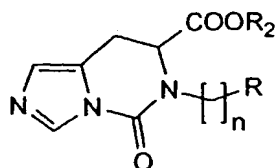


Histidine derivatives can also be involved in the peptide chain without reversing the sequence, such as in the following normal sequence with a bidentate histidine ligand in the sequence:



In this case it is possible to include a bidentate, natural ligand in a normal peptide sequence. This inclusion in the peptide chain yields a novel kind of labeling which has not been realized so far.

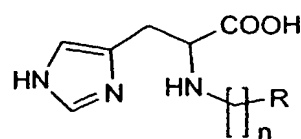
Modification at  $N^{\alpha}$  in structure yields a semi-natural histidine. Derivatization at either  $N^{\alpha}$  or  $N^{\epsilon}$  is selectively possible as follows:



$R = \text{NH}_2, \text{COOR}_1, \text{OH}, \text{X}$   
 $R_1 = \text{H}, t\text{-butyl}$   
 $R_2 = \text{H}, \text{methyl}$

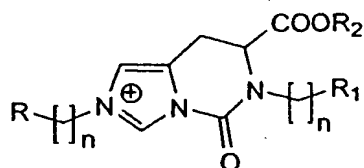
5

modification



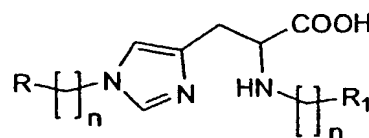
deprotection

Derivatization at both  $N^{\alpha}$  and  $N^{\epsilon}$  is possible at the same time, introducing different functionalities (including also biomolecules) (left) to yield a trifunctional histidine after deprotection, which then gives a trifunctionalized tripodal histidine ligand (right):



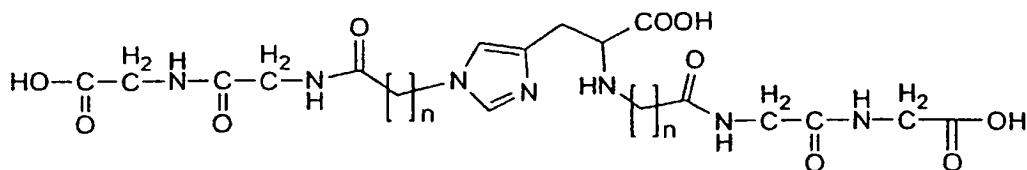
$R = \text{NH}_2, \text{COOH}, \text{OH}, \text{X}$   
 $R_1 = \text{NH}_2, \text{COOH}, \text{OH}, \text{X}$   
 $R_2 = \text{H}, \text{methyl}$

15

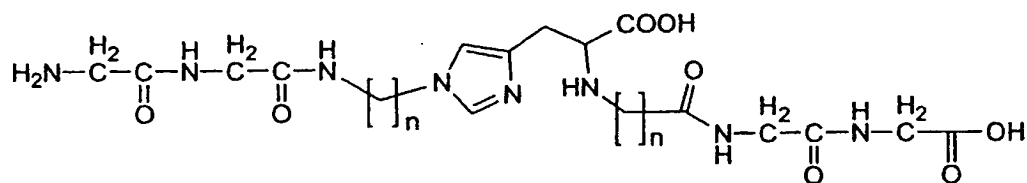


Alternatively, coupling of peptide sequences in either  $R$  or  $R_1$  allows the inclusion of tridentate histidine in a peptide sequence which can be normal (bottom) or reversed (top).

15



tripodal histidine



tripodal histidine

Both strategies allow also the labeling of small molecules by applying the highly efficient and strong histidine ligand. This can be used for i.e. the labeling of amino acids or other small molecules such as hypoxia imaging agents. The  $[Tc(his-R)(CO)_3]$  complex is thereby of high hydrophilicity which is in general an advantage for biological molecules.

It should be noted that in this application the compounds as depicted in structural formulas I to XVIII are generalized structures. The following Table 1 summarizes the combination of features for each general formula.

Table 1

	A	B	C	D
1	II/XIII	IX/X	III/XI XVIII/XI	III, XI
2	IV/-	np/np	V/XVI	V, XVI
3	VI/-	np/np	VII/XVII	VII, XVII
4	I/XIV	XII/XV	-	histidine



- 1 = derivatized at N<sup>c</sup>  
 2 = derivatized at N<sup>a</sup>  
 3 = derivatized at N<sup>c</sup> and N<sup>a</sup>  
 5 4 = not derivatized  
 A = protected by means of six-membered ring  
 B = protected by means of metal tricarbonyl  
 C = protected by ester on COOH and amine protecting group on N<sup>a</sup>  
 D = formula with deprotected COOH and N<sup>a</sup> from column C for labeling  
 10 **bold** = formula without biomolecule  
*italic* = formula with biomolecule  
 np = not possible

It is the applicant's intention to disclose herein all  
 15 possible combinations of the alkyl chain length expressed in  
 the value of n and the various substituents R on N<sup>a</sup> and/or  
 N<sup>c</sup>. The following is Table 2 in which all possible  
 combinations of n and R are listed. In some of the compounds  
 any one combination of two of the combinations listed below  
 20 are possible.

Table 2

	0	1	2	3	4	5	6	7	8	9	10
NH <sub>2</sub>	0/NH <sub>2</sub>	1/NH <sub>2</sub>	2/NH <sub>2</sub>	3/NH <sub>2</sub>	4/NH <sub>2</sub>	5/NH <sub>2</sub>	6/NH <sub>2</sub>	7/NH <sub>2</sub>	8/NH <sub>2</sub>	9/NH <sub>2</sub>	10/NH <sub>2</sub>
COOH	0/ COOH	1/ COOH	2/ COOH	3/ COOH	4/ COOH	5/ COOH	6/ COOH	7/ COOH	8/ COOH	9/ COOH	10/ COOH
OH	0/OH	1/OH	2/OH	3/OH	4/OH	5/OH	6/OH	7/OH	8/OH	9/OH	10/OH
X	0/X	1/X	2/X	3/X	4/X	5/X	6/X	7/X	8/X	9/X	10/X

When the biomolecule is not coupled directly to the histidine  
 25 but when the N<sup>c</sup> and/or N<sup>a</sup> are derivatized first, the  
 following combinations with n and R are possible.

Table 3

	0	1	2	3	4	5	6	7	8	9	10
NH <sub>2</sub>	0/NH-BM	1/NH-BM	2/NH-BM	3/NH-BM	4/NH-BM	5/NH-BM	6/NH-BM	7/NH-BM	8/NH-BM	9/NH-BM	10/NH-BM
COOH	0/CO-BM	1/CO-BM	2/CO-BM	3/CO-BM	4/CO-BM	5/CO-BM	6/CO-BM	7/CO-BM	8/CO-BM	9/CO-BM	10/CO-BM
OH	0/O-BM	1/O-BM	2/O-BM	3/O-BM	4/O-BM	5/O-BM	6/O-BM	7/O-BM	8/O-BM	9/O-BM	10/O-BM
X	0/X'-BM	1/X'-BM	2/X'-BM	3/X'-BM	4/X'-BM	5/X'-BM	6/X'-BM	7/X'-BM	8/X'-BM	9/X'-BM	10/X'-BM

NH-BM means that the NH<sub>2</sub> on the N<sup>ε</sup> and/or N<sup>α</sup> of the histidine derivative is coupled to the COOH of the biomolecule. In CO-BM the COOH on the N<sup>ε</sup> and/or N<sup>α</sup> of the histidine derivative is coupled to the NH<sub>2</sub> of the biomolecule. O-BM means that the OH on the N<sup>ε</sup> and/or N<sup>α</sup> of the histidine derivative is coupled to the Ph-OH or halide on the biomolecules by the formation of a phosphate-ester or an ether linking group of the biomolecule. And X'-BM means that the halide, azide, pseudohalide, phosphate, thiol or silyl on the N<sup>ε</sup> and/or N<sup>α</sup> of the histidine derivative is coupled to S, OH or amine on the biomolecule.

The invention will be further illustrated in the Examples that follows and that is not intended to limit the invention in any way. The Examples describe a model system for the labeling of biomolecules. It should be noted that in the same manner other biomolecules, such as amino acids and peptides can be labeled. In the Example reference is made to the following figures:

**Figure 1:** Ortep plot of 3, ellipsoids drawn at 50% probability.

**Figure 2:** Ortep plot of **6**, ellipsoids drawn at 50% probability, showing one of the two molecules in the asymmetric unit.

**Figure 3:** Ortep plot of **13**, ellipsoids drawn at 50% probability, showing one of the two molecules in the asymmetric unit.

### **EXAMPLES**

#### **EXAMPLE 1**

#### **Synthesis of N<sup>α</sup> and N<sup>δ</sup> protected urea-histidine (Scheme 1)**

##### **5-Oxo-5,6,7,8-tetrahydroimidazo[1,5-c]pyrimidine-7-carboxylic acid methyl ester (molecule 3)**

The compound was prepared according to literature with slight modification. (R. Jain et al. Tetrahedron, 1996, 52, 5363) To a solution of L-histidine methylester (2.73g, 11.28 mmol) in DMF (80 ml) was added Im<sub>2</sub>CO (1.88g, 11.61 mmol) at r.t. The reaction mixture was heated to 70°C for 6 h, cooled down to r.t. and poured slowly to 1M NaHCO<sub>3</sub> aqueous solution (250 ml). Some solid precipitated from the water layer, which was extracted with CH<sub>2</sub>Cl<sub>2</sub>. During extraction the precipitate dissolved completely in CH<sub>2</sub>Cl<sub>2</sub>.

The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub>, concentrated under reduced pressure, and purified by flash column chromatography to afford **3** as white solid (1.35 g, 61%). R<sub>f</sub> = 0.2 (EtOAc 100%); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>CN, 25°C): δ = 8.01 (s, 1H; CH<sub>im</sub>), 6.77 (s, 1H; CH<sub>im</sub>), 6.61 (br.s, 1H; NH), 4.37-4.34 (m, 1H; CHCO), 3.67 (s, 3H; OCH<sub>3</sub>), 3.25-3.23 (m, 2H; CH<sub>2</sub>CH); <sup>13</sup>C NMR (500 MHz, CD<sub>3</sub>CN, 25°C): δ = 172.1, 149.2, 135.4, 126.9, 125.9, 53.6, 53.5, 23.7; MS (ESI): m/z (%): 195.73 (100) [M<sup>+</sup>], 167.8 (35), 135.8 (24); elemental analysis

calcd (%) for  $C_8H_9N_3O_3$  (195.18): C 49.23, H 4.62, N 21.54;  
found: C 49.32, H 4.77, N 21.24.

Crystals suitable for x-ray structure analysis were  
obtained by slow evaporation from EtOAc.

5

## EXAMPLE 2

Introduction of carboxylate functionality in N<sup>ε</sup> (Scheme 1)  
**2-(2-tert-Butoxy-2-oxoethyl)-7-methoxycarbonyl-5-oxo-5,6,7,8-**  
**tetrahydroimidazo[1,5-c]pyrimidine-2-ium;bromide (molecule 4)**

10 Bromoacetic acid tert-butyl ester (0.57 ml, 3.86 mmol)  
was added to a solution of **3** (250 mg, 1.28 mmol) in  $CH_3CN$  (25  
ml). The reaction mixture was refluxed for 24 h, cooled to  
r.t. and concentrated *in vacuo*. The residue was washed with  
Et<sub>2</sub>O (2 x 10 ml) and THF (2 x 5 ml) and dried *in vacuo* to  
15 afford **4** as a white sticky solid, which was used in the next  
step without any further purification. <sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O,  
20°C): δ = 9.39 (s, 1H; CH<sub>im</sub>), 7.40 (s, 1H; CH<sub>im</sub>), 5.05 (s, 2H;  
CH<sub>2</sub>N<sub>im</sub>), 4.78-4.61 (m, 1H; CHCO), 3.63 (s, 3H; OCH<sub>3</sub>), 3.42-  
3.39 (m, 2H; CH<sub>2</sub>CH), 1.39 (s, 9H; tBu); MS (ESI): m/z (%):  
20 309.40 (13) [M<sup>+</sup>-HBr], 253.80 (100) [M<sup>+</sup>-HBr-(CH<sub>2</sub>=C(CH<sub>3</sub>)<sub>2</sub>)].

## EXAMPLE 3

Preparation of N<sup>ε</sup> protected histidine (Scheme 1)

**Methyl N-[(benzyloxy) carbonyl]-1-(2-tert-butoxy-2-oxoethyl)**  
25 **histidinate (molecule 6)**

To a solution of crude **4** (390 mg) in THF (50 ml) were  
added DIPEA (0.52 ml, 3.01 mmol) and BnOH (2.1 ml, 20.08  
mmol). After 16 h of refluxing, the reaction solution was  
cooled down to room temperature, concentrated under reduced  
30 pressure, and purified by flash column chromatography to  
afford **6** as white solid (260 mg, 62% from **3**). R<sub>f</sub> = 0.15  
(CH<sub>2</sub>Cl<sub>2</sub>/MeOH 45:1); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>CN, 25°C): δ = 7.39-

7.32 (m, 6H; 5 x CH<sub>ph</sub>, CH<sub>im</sub>), 6.78 (s, 1H; CH<sub>im</sub>), 6.66 (br.d,  $J$  = 7.8 Hz, 1H; NH), 5.06 (s, 2H; CH<sub>2</sub>-Bn), 4.58 (s, 2H; CH<sub>2</sub>N<sub>im</sub>), 4.42 (q,  $J$  = 2.6 Hz, 1H; CHCO), 3.62 (s, 3H; OCH<sub>3</sub>), 2.96 (t,  $J$  = 5.26 Hz, 2H; CH<sub>2</sub>CH); <sup>13</sup>C NMR (500 MHz, CD<sub>3</sub>CN, 25°C):  $\delta$  =  
5 173.2, 168.3, 157.0, 139.1, 138.2, 137.8, 129.5, 129.0, 128.9, 119.2, 83.2, 67.2, 66.9, 55.2, 52.7, 49.3, 30.2, 28.2;  
MS (ESI):  $m/z$  (%): 417.53 (100) [M<sup>+</sup>]; elemental analysis  
calculated (%) for C<sub>21</sub>H<sub>27</sub>N<sub>3</sub>O<sub>6</sub> (417.48): C 60.43, H 6.47, N  
10.07; found: C 60.43, H 6.57, N 9.97.

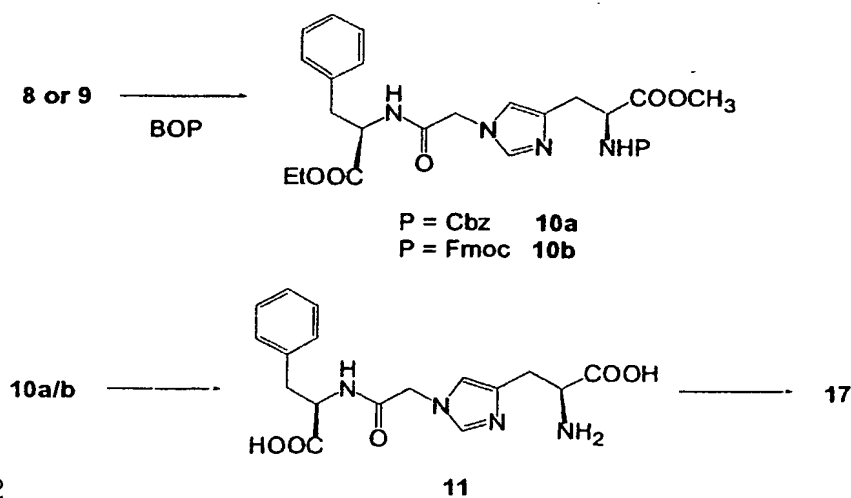
10 Crystals suitable for x-ray structure analysis were  
obtained by vapor diffusion of 1-hexene into EtOAc.

#### EXAMPLE 4

Preparation of a histidine compound with deprotected  
15 functionality and coupling to a biomolecule (Scheme 2)  
**Methyl N-[(benzyloxy)carbonyl]-1-{2-[(1-ethoxycarbonyl-2-  
phenylethyl)amino]-2-oxoethyl} histidinate (molecule 10a)**

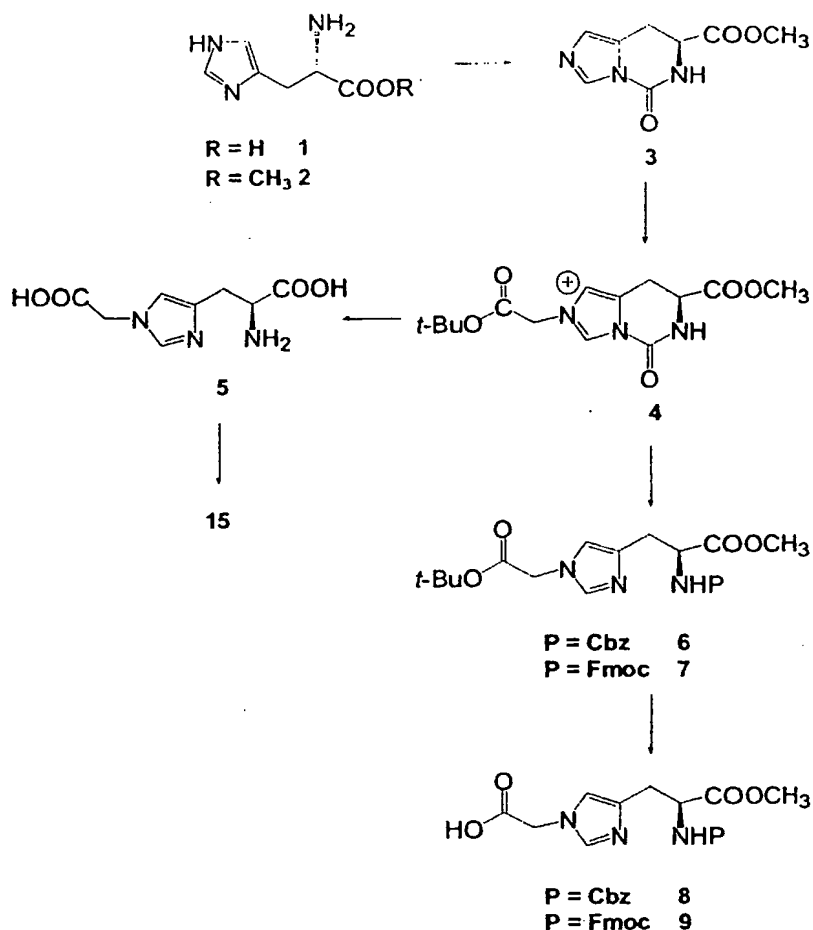
A solution of **6** (140 mg, 0.34 mmol) in CH<sub>2</sub>Cl<sub>2</sub>/TFA (2:2  
ml) was stirred for 2.5 h at r.t. The solvent was removed  
20 under reduced pressure and dried more *in vacuo*. The residue,  
the crude compound **8** was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (10 ml) and  
neutralized by adding Et<sub>3</sub>N dropwise. BOP (148 mg, 0.34 mmol)  
and Et<sub>3</sub>N (46  $\mu$ l, 0.34 mmol) were added to the reaction  
mixture. After 45 min, a solution of phenylalanine-ethyl  
25 ester (84.6 mg, 0.37 mmol) and Et<sub>3</sub>N (51  $\mu$ l, 0.37 mmol) in  
CH<sub>2</sub>Cl<sub>2</sub> (10 ml) was added slowly by a syringe. The reaction  
mixture was stirred for 2.5 d more at room temperature. The  
solution was diluted with CH<sub>2</sub>Cl<sub>2</sub> (30 ml) and extracted with  
1N HCl solution (20 ml), 1N NaHCO<sub>3</sub> (20 ml), brine (20 ml).  
30 The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, concentrated under  
reduced pressure and purified by flash column chromatography  
to afford **10a** as colorless oil (162 mg, 90%).  $R_f$  = 0.2

(CH<sub>2</sub>Cl<sub>2</sub>/MeOH 40:1); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>CN, 25°C): δ = 7.37-7.28 (m, 9H; 2 x 4H-CH<sub>ph</sub>, CH<sub>im</sub>), 7.16 (d, *J* = 8.23 Hz, 2H; 2 x CH<sub>ph</sub>), 6.77 (br.d, *J* = 7.65 Hz, 1H; NH), 6.69-6.66 (m, 2H; CH<sub>im</sub>, NH), 5.05 (s, 2H; CH<sub>2</sub>-Bn), 4.61 (dt, *J* = 7.86 Hz, 1H; CH-Phe), 4.52 (s, 2H; CH<sub>2</sub>N<sub>im</sub>), 4.43-4.41 (m, 1H; CHCO), 4.11 (q, *J* = 7.15 Hz, 2H; CH<sub>2</sub>CH<sub>3</sub>), 3.62 (s, 3H; OCH<sub>3</sub>), 3.10 (dd, *J* = 8.19 Hz, 1H; CH<sub>2</sub>-Phe), 2.99-2.93 (m, 3H; CH<sub>2</sub>-Phe, CH<sub>2</sub>CH), 2.57 (s, N-CH<sub>3</sub>), 1.18 (t, *J* = 7.13 Hz, 3H; CH<sub>3</sub>); <sup>13</sup>C NMR (500 MHz, CD<sub>3</sub>CN, 25°C): δ = 173.3, 172.0, 167.8, 157.0, 139.1, 138.3, 138.2, 137.8, 130.4, 129.5, 129.4, 129.0, 128.9, 127.9, 118.9, 67.2, 62.2, 55.2, 54.8, 52.8, 49.9, 38.0, 30.3, 14.5; MS (ESI): *m/z* (%): 537.53 (100) [M<sup>+</sup>+H]; elemental analysis calcd (%) for C<sub>28</sub>H<sub>32</sub>N<sub>4</sub>O<sub>7</sub>+0.5[N(CH<sub>3</sub>)<sub>2</sub>]<sub>3</sub>P=O+0.5H<sub>2</sub>O (634.5): C 58.63, H 6.62, N 12.14; found: C 58.98, H 6.89, N 12.48.



Scheme 2

22



Scheme 1

5

**EXAMPLE 5**

Introduction of a functional group at N<sup>ε</sup> in [Re(CO)<sub>3</sub>]<sup>+</sup> protected histidine (Scheme 3)

**Re complex (16a)**

- 10 To a solution of complex **14**, (25 mg, 0.059 mmol) and Cs<sub>2</sub>CO<sub>3</sub> (20.4 mg, 0.065 mmol) in acetonitrile (25 ml) ethyl bromoacetate (29.5 mg, 0.176 mmol) in acetonitrile (5 ml) was added. The reaction mixture was heated at 35°C for 1.5 h. Glacial acetic acid was added to the mixture to neutralize.
- 15 After standard work-up, the crude substance was purified by a

silica gel chromatography to provide complex **16a** (30 mg, 90%).  $R_f = 0.15$  (EtOAc/EtOH 5:1);  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{CN}$ ,  $20^\circ\text{C}$ ):  $\delta = 7.95$  (s, 1H;  $\text{CH}_{\text{im}}$ ), 6.92 (s, 1H;  $\text{CH}_{\text{im}}$ ), 4.78 (s, 2H;  $\text{CH}_2\text{N}_{\text{im}}$ ), 4.23-4.16 (q,  $J = 7.1$  Hz, 2H;  $\text{CH}_2\text{CH}_3$ ), 3.91-3.87 (m, 1H;  $\text{CHCO}$ ), 3.21-2.98 (q, 2H;  $\text{CH}_2\text{CH}$ ), 1.28-1.23 (t,  $J = 7.5$  Hz, 3H;  $\text{CH}_3$ );  $^{13}\text{C}$  NMR (300 MHz,  $\text{CD}_3\text{CN}$ ,  $20^\circ\text{C}$ ):  $\delta = 199.5$ , 197.8, 197.8, 181.8, 168.8, 143.4, 135.7, 120.9, 63.0, 52.6, 49.4, 28.7, 14.4; MS (ESI):  $m/z$  (%): 511.8 (100) [ $\text{M}^+ + \text{H}$ ], 1020.7 (55) [ $2\text{M}^+$ ]; elemental analysis calcd (%) for  $\text{C}_{15}\text{H}_{18}\text{N}_3\text{O}_7\text{Re}$  (510.5): C 30.59, H 2.76, N 8.23; found: C 30.84, H 3.0, N 8.06.

#### Re complex (16b)

The preparation is similar to compound **16a**. To compound **14**, (25 mg, 0.059 mmol) and  $\text{Cs}_2\text{CO}_3$  (20.4 mg, 0.065 mmol) in acetonitrile (25 ml) was added *tert*-butyl bromoacetate (34.5 mg, 0.176 mmol). The reaction mixture was stirred at  $35^\circ\text{C}$  for 1.5 h. The reaction mixture was filtered, dried under vacuum and purified by silica gel chromatography (EtOAc/EtOH 5:1) to yield complex **16b** (29 mg, 90%).  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{CN}$ ,  $20^\circ\text{C}$ )  $\delta = 7.93$  (s, 1H;  $\text{CH}_{\text{im}}$ ), 6.90 (s,  $\text{CH}_{\text{im}}$ ), 4.65 (s, 2H;  $\text{CH}_2\text{N}_{\text{im}}$ ), 3.94-3.25 (m, 1H;  $\text{CHCO}$ ), 3.27-3.23 (q, 2H;  $\text{CH}_2\text{CH}$ ), 1.45 (s, 9H; *t*Bu);  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{CN}$ )  $\delta = 181.3$ , 167.3, 143.1, 135.2, 120.6, 83.8, 52.3, 49.7, 28.5, 28.0, 27.8; MS (ESI):  $m/z$  (%): 539.9 (100) [ $\text{M}^+$ ], 1076.8 (50); elemental analysis calcd (%) for  $\text{C}_{15}\text{H}_{18}\text{N}_3\text{O}_7\text{Re}$  (538.5): C 33.43, H 3.34, N 7.80; found: C 33.30, H 3.85, N 7.68.

#### Re complex 15

To hydrolyze the ester groups, compound **16a** (30mg, 0.057 mmol) was stirred in a solution of methanol (5 mL) and LiOH (0.5 M, 2 ml) overnight at room temperature and compound **16b**



(15 mg, 0.028 mmol) was stirred in a solution of methylene chloride (2 ml) and trifluoroacetic acid (2 ml) for 2 hours at room temperature. Two crude substances were purified by column chromatography (EtOH/THF/AcOH 10:1:0.1) to yield  
5 complex **15** (95% and 90% respectively).

#### EXAMPLE 6

Coupling of a biomolecule to the carboxylate group in  
 $[\text{Re}(\text{CO})_3]^+$  protected histidine and removal of the  $[\text{Re}(\text{CO})_3]^+$   
10 protecting group (Scheme 4)

#### Re complex **17**

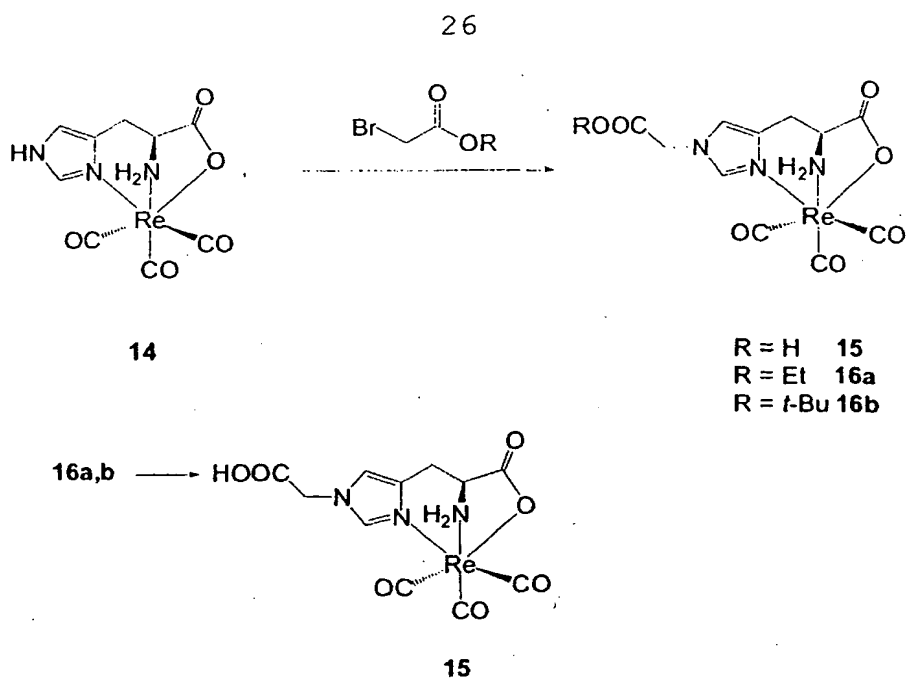
To the solution of the complex **15** (8 mg, 0.02 mmol) in a mixed solution of  $\text{CH}_2\text{Cl}_2/\text{DMF}$  (3:0.2 ml) were added BOP (7.4 mg, 0.02 mmol) and  $\text{Et}_3\text{N}$  (2  $\mu\text{l}$ , 0.02 mmol) at room  
15 temperature. After 30 min, a solution of phenylalanine-ethyl ester (4 mg, 0.02 mmol) and  $\text{Et}_3\text{N}$  (2  $\mu\text{l}$ , 0.02 mmol) in  $\text{CH}_2\text{Cl}_2$  (2 ml) was added dropwise to the complex solution by syringe. The reaction mixture was stirred overnight. The reaction solution was concentrated in vacuo. The residue was treated  
20 with diethyl ether (2 x 5 ml). The white solid was dissolved in THF (10 ml) and insoluble solid was filtered off. The filtrate was concentrated in vacuo to provide the ethyl ester of complex **17** (75%).  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{CN}$ , 25°C):  $\delta$  = 7.8 (s, 1H;  $\text{CH}_{\text{im}}$ ), 7.34-7.21 (m, 5H;  $\text{CH}_{\text{ph}}$ ), 6.8 (s, 1H;  $\text{CH}_{\text{im}}$ ),  
25 4.63-4.59 (m, 1H;  $\text{CHCO}$ ), 4.53 (d, 2H;  $\text{CH}_2\text{N}_{\text{im}}$ ), 4.08 (q, 2H;  $\text{CH}_2\text{CH}_3$ ), 3.92-3.88 (m, 1H;  $\text{CH-His}$ ), 3.18-3.10 (2 x dd, 2H;  $\text{CH}_2\text{-His}$ ,  $\text{CH}_2\text{-Phe}$ ), 3.05-2.96 (2 x dd, 2H;  $\text{CH}_2\text{-His}$ ,  $\text{CH}_2\text{-Phe}$ ), 1.17 (t, 3H;  $\text{CH}_3$ );  $^{13}\text{C}$  NMR (500 MHz,  $\text{CD}_3\text{CN}$ , 25°C):  $\delta$  = 198.1, 196.6, 196.5, 180.4, 170.8, 165.5, 142.0, 136.7, 134.3,  
30 129.4, 128.4, 126.8, 124.9, 120.3, 119.6, 61.2, 54.1, 51.4, 49.2, 37.2, 27.7, 13.4; IR (KBr):  $\bar{\nu}$  = 2020, 1886, 1733, 1636  $\text{cm}^{-1}$ ; MS (ESI):  $m/z$  (%): 659 (100) [ $\text{M}^+ + \text{H}$ ]; elemental analysis

calcd (%) for  $C_{22}H_{24}N_4O_8Re$  (658.6): C 40.12, H 3.67, N 8.51;  
found: C 39.31, H 3.83, N 8.25.

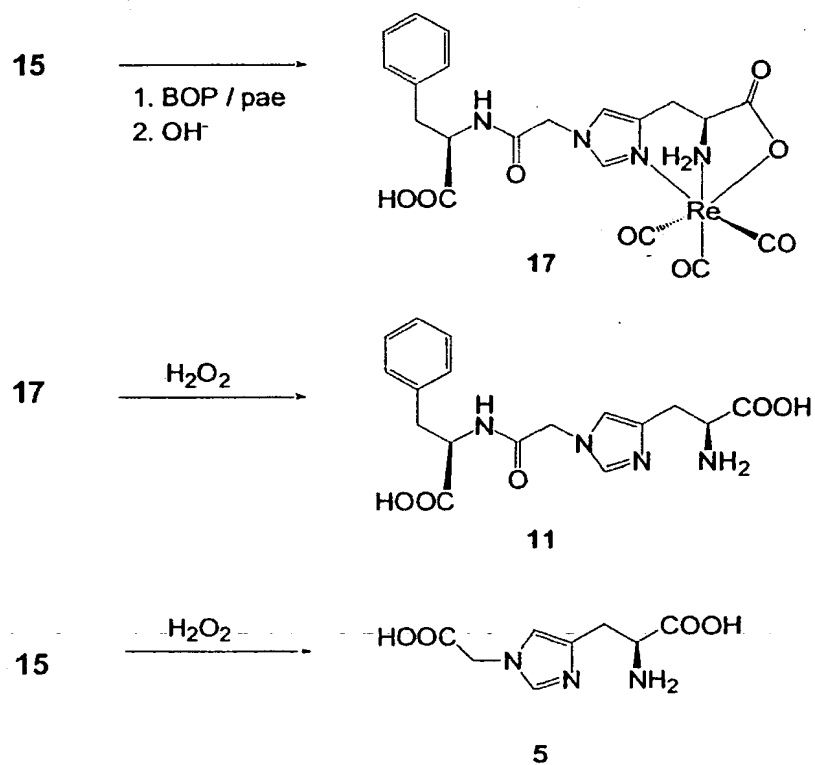
Ethyl ester group of the complex was hydrolyzed by stirring the complex in mixed solution of 0.5M LiOH and MeOH (1:2) for overnight at room temperature, as mentioned above, to afford complex 17 quantitatively.

#### General Procedure for the Oxidation of Rhenium from Complex 17 and 15

10 A solution of compound 17 or 15 (5 mM in  $H_2O$ , 500  $\mu$ l) and acid (HCl, TFA, or acetic) solution (1.0, 0.1, or 0.01M in  $H_2O$ , 70  $\mu$ l) were added to a vial, which was sealed then degassed with nitrogen (10 min).  $H_2O_2$  (0.43, 0.86, or 1.29M in  $H_2O$ , 60  $\mu$ l) was added to the degassed vial, followed by  
15 heating the sample at 50°C. Monitoring of the reaction mixture was conducted by HPLC at 250 nm, where the reaction mixture (10  $\mu$ L) was injected on the HPLC at 4, 8, 24, and 48 hrs or until the rhenium complex was not visible in the spectrum. The effectiveness of the reaction condition was  
20 calculated by determining the peak area ratio of the rhenium complex over the formation of perrhenate. When the rhenium complex was no longer observed, the reaction mixture was treated with manganese dioxide to remove residual  $H_2O_2$  from the reaction mixture then filtered with a Wattman 0.2  $\mu$ m  
25 filter to yield the uncoordinated ligand in solution to be used in  $^{99m}Tc$  labeling.



Scheme 3



5 Scheme 4

**EXAMPLE 7**

Preparation of histidine with an N<sup>c</sup> pendant -NH<sub>2</sub> group  
(Scheme 5)

**3-[1-[3-(9H-Fluoren-9-ylmethoxycarbonylamino)-propyl]-1H-imidazol-4-yl]-2-(3-trimethylsilyl-propionylamino)-propionic acid methyl ester (molecule 18)**

A mixture of **3** (196 mg; 1.0 mmol) and N-Fmoc-3-iodopropylamine (1.22 g; 3.0 mmol) in MeCN (40 ml) was heated at reflux for 4.5 days. When compound **3**, urea derivative, was not detectable by TLC, the reaction mixture was concentrated *in vacuo*, resulting in a white solid. The solid material was redissolved in MeCN (40 ml) and 2-trimethylsilylethanol (355 mg; 3.0 mmol) and dipea (259 mg; 2.0 mmol) were added. The resulting mixture was stirred at RT under N<sub>2</sub> for 16 hrs. The solvent was removed *in vacuo*, followed by purification by column chromatography (silica; EtOAc). Yield: 316 mg (53% over two steps) of a foamy colourless solid

Found: C, 62.1; H, 6.2; N, 9.5; Calc. for C<sub>31</sub>H<sub>40</sub>N<sub>4</sub>O<sub>6</sub>Si: C, 62.8; H, 6.8; N 9.5;  $\nu_{\max}$  (KBr)/cm<sup>-1</sup> 3329br NH, 1730s, 1698vs C=O;  $\delta_{\text{H}}$  (300.8 MHz; CD<sub>3</sub>CN) 7.82 (2H, pseudo-d, 2 × ArH), 7.64 (2H, pseudo-d, 2 × ArH), 7.38 (3H, m, 2 × ArH + N<sub>2</sub>CH<sub>His</sub>), 7.32 (2H, pseudo-t, 2 × ArH), 6.79 (1H, s, CH<sub>His</sub>), 6.55 (1H, d, *J* 7.5, NH), 5.68 (1H, br s, NH), 4.35 (3H, overlapping m, OCH<sub>2</sub>-Fmoc + C <sub>$\alpha$</sub> H), 4.22 (1H, t, *J* 6.6, OCH<sub>2</sub>CH-Fmoc), 4.07 (2H, m, CH<sub>2</sub>), 3.87 (2H, m, CH<sub>2</sub>), 3.60 (3H, s, OCH<sub>3</sub>), 2.99 (2H, m, CH<sub>2</sub>), 2.90 (2H, m, C <sub>$\beta$</sub> H<sub>2</sub>), 1.85 (2H, m, CH<sub>2</sub>), 0.93 (2H, m, CH<sub>2</sub>), 0.01 (s, 9H, Si-(CH<sub>3</sub>)<sub>3</sub>).  $\delta_{\text{C}}$  (CD<sub>3</sub>CN; 75.47 MHz) 173.7 (C=O<sub>ester</sub>), 157.7, 157.6 (2 × C=O<sub>amide</sub>), 145.5, 142.2 (2 × ArC<sub>q</sub>), 138.4 (C<sub>His</sub>), 128.9, 128.3 (2 × ArCH), 126.4 (C<sub>His</sub>), 121.2, 118.6 (2 × ArCH), 118.1 (C<sub>His</sub>), 66.9, 63.7, 55.3, 52.7, 48.3,

44.9, 38.6, 32.1, 30.1, 18.3, 1.4 (Si(CH<sub>3</sub>)<sub>3</sub>); *m/z* (ESI-pos., MeOH) 343, 371, 533, 593 [M+H]<sup>+</sup>.

3-[1-(3-amino-propyl)-1*H*-imidazol-4-yl]-2-(3-trimethyl  
5 silanyl-propionylamino)-propionic acid methyl ester (molecule 19)

Compound **18** (255 mg; 0.43 mmol) was dissolved in a 1/1 DMF/NEt<sub>2</sub> mixture (8 ml). After the mixture was stirred for 1 hr at RT, the solvent was removed *in vacuo*. Purification by  
10 preparative HPLC (C-18ec column; TFA buffer) afforded compound **19** as a colourless foamy solid as its trifluoroacetate salt. Yield: 190 mg (91%).

$\delta_H$  (300.08 MHz; CD<sub>3</sub>CN) 8.59 (1H, s, N<sub>2</sub>CH<sub>His</sub>), 7.95 (3H, br, NH<sub>3</sub><sup>+</sup>), 7.26 (1H, s, CH<sub>His</sub>), 6.56 (1H, d, *J* 8.4 Hz, NH), 4.43  
15 (1H, m, C <sub>$\alpha$</sub> H), 4.24 (2H, t, *J* 6.9 Hz, CH<sub>2</sub>), 4.05 (2H, m, CH<sub>2</sub>), 3.68 (3H, s, OCH<sub>3</sub>), 3.22 (1H, m, C <sub>$\beta$</sub> H), 3.06 (1H, m, C <sub>$\beta$</sub> H), 2.95 (2H, t, *J* 6.9, CH<sub>2</sub>), 2.21 (2H, m, CH<sub>2</sub>), 0.89 (2H, m, CH<sub>2</sub>), 0.00 (9H, s, Si-(CH<sub>3</sub>)<sub>3</sub>);  $\delta_C$  (75.47 MHz; CD<sub>3</sub>CN): 172.7 (C=O<sub>ester</sub>), 162.2 (q, *J*<sub>C,F</sub> 34.6, CF<sub>3</sub>), 157.7 (C=O<sub>amide</sub>), 135.9,  
20 132.4, 120.7 (3 × C<sub>His</sub>), 64.0, 54.6, 53.2, 47.0, 37.3, 28.6, 27.6, 18.2 (OCH<sub>3</sub>, C <sub>$\alpha$</sub> , C <sub>$\beta$</sub>  + 5 × CH<sub>2</sub>), 1.5 (Si-(CH<sub>3</sub>)<sub>3</sub>); *m/z* (ESI-pos.; MeOH): 343.1, 370.8 ([M+H]<sup>+</sup>, C<sub>16</sub>H<sub>30</sub>N<sub>4</sub>O<sub>4</sub>Si requires 371.2) 762 [2M+Na]<sup>+</sup>.

25 **EXAMPLE 8**

Coupling of biomolecules to amino group in histidine derivative (Scheme 5)

a) **biotin**: D-(+)-Biotin (35 mg; 0.14 mmol) was dissolved in a 4/1 (v/v) mixture of DMF/NEt<sub>3</sub> (2.5 ml). To this mixture was  
30 added a solution of compound **19** (91 mg; 0.19 mmol) in DMF (2 ml), followed by addition of TBTU (46 mg; 0.14 mmol). After the mixture was stirred for 45 min at RT, it was evaporated

to dryness *in vacuo*. The residue was taken up in 2M NaHCO<sub>3</sub> (20 ml) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (3×20 ml). The combined organic layers were washed with 0.5 M HCl (20 ml), H<sub>2</sub>O (20 ml) and brine (2 × 20 ml). After removal of the solvent, compound 20 was obtained as a colourless foamy solid. Yield: 67 mg (78%; relative to biotin).

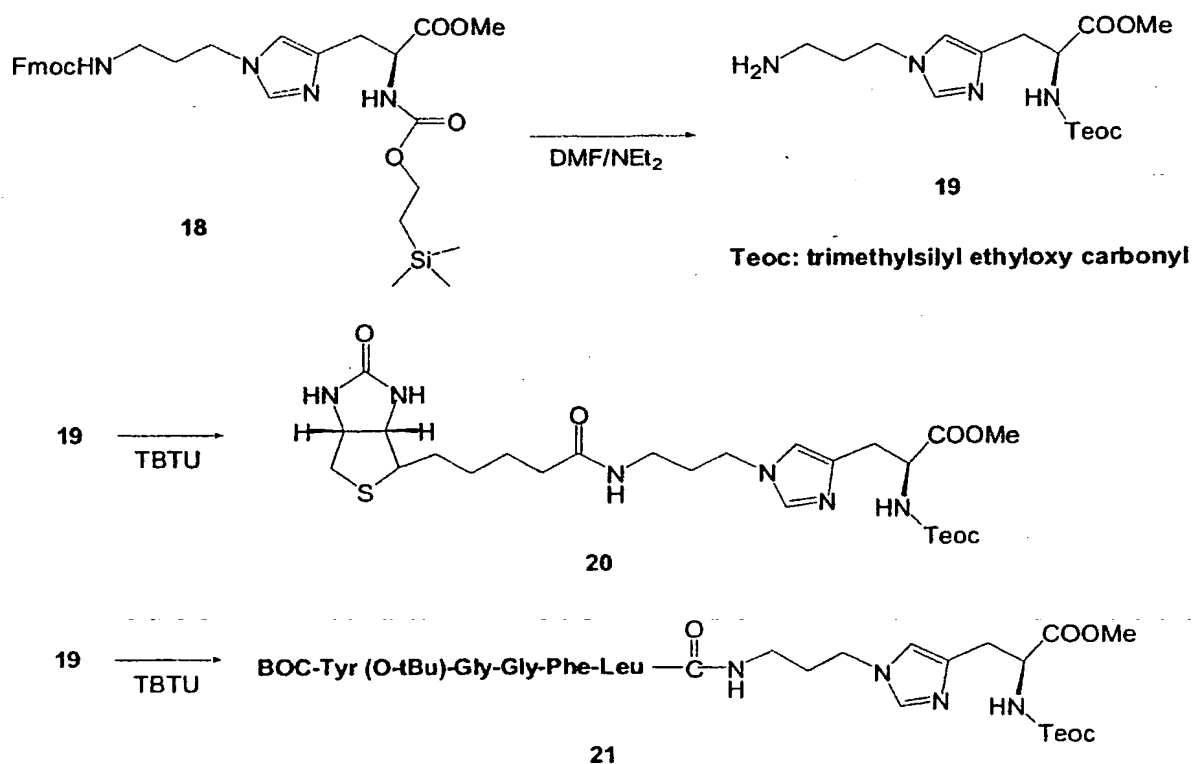
$\delta_H$  (300.08 MHz; CD<sub>3</sub>OD) 7.60 (1H, s, N<sub>2</sub>CH<sub>His</sub>), 6.95 (1H, s, CH<sub>His</sub>), 4.48 (1H, m), 4.41 (1H, m), 4.29 (1H, m), 4.09 (2H, m), 3.98 (2H, m), 3.69 (3H, s, OCH<sub>3</sub>), 3.16 (3H, overlapping-m, CH<sub>2</sub> + H), 2.95–2.78 (5H, overlapping-m, 2 × CH<sub>2</sub> + H), 2.70 (1H, m), 2.19 (2H, m, CH<sub>2</sub>), 1.93 (2H, m, CH<sub>2</sub>), 1.63 (4H, m, 2 × CH<sub>2</sub>), 1.43 (2H, m, CH<sub>2</sub>), 0.94 (2H, m, CH<sub>2</sub>), 0.01 (9H, s, Si-(CH<sub>3</sub>)<sub>3</sub>);  $\delta_C$  (75.47 MHz; CD<sub>3</sub>OD): 176.4, 174.3 (C=O<sub>ester</sub> + N<sub>2</sub>C=O), 166.3, 158.9 (C=O<sub>amide</sub>), 138.5, 138.3, 118.7 (ArCH<sub>His</sub>), 64.2, 64.4, 61.7, 57.1, 55.7, 52.8, 45.7, 41.1, 37.4, 36.8, 31.9, 31.1, 29.8, 29.5, 26.8, 18.6 (10 × CH<sub>2</sub>, C $\alpha$ , C $\beta$ , OCH<sub>3</sub>, 3 × CH), 1.5 (Si-(CH<sub>3</sub>)<sub>3</sub>); *m/z* (FAB<sup>+</sup>; NBA) 597.2898 (M<sup>+</sup>, C<sub>26</sub>H<sub>45</sub>N<sub>6</sub>O<sub>6</sub>SiS requires 597.2891).

**b) to enkephalin:** To a solution of purified compound 19 (39 mg; 0.08 mmol) and protected enkephalin (85 mg; 0.08 mmol) in DMF (1.5 ml) and NEt<sub>3</sub> (0.5 ml) was added TBTU (25 mg; 0.08 mmol). The mixture was stirred for 45 mins at RT and concentrated to dryness *in vacuo*. The compound was purified by HPLC (run 1: C8-column; 50 mM TRIS buffer; run 2: C8-column; TFA buffer). Yield: 42 mg (49%) of compound 21 as glassy solid.

$\delta_H$  (500.25 MHz; CD<sub>3</sub>OD) 8.77 (1H, s, N<sub>2</sub>CH<sub>His</sub>), 7.43 (1H, s, CH<sub>His</sub>), 7.28 (4H, m, 4 × ArH), 7.20 (1H, m, ArH), 7.12 (2H, pseudo-d, 2 × ArH), 6.90 (2H, pseudo-d, 2 × ArH), 4.52 (1H, m, C $\alpha$ H), 4.49 (1H, m, C $\alpha$ H), 4.22–4.09 (7H, overlapping m, 2 ×

$C_{\alpha}H + 2 \times CH_2$ ), 3.85 (2H, m,  $CH_2$ -Gly), 3.78 (2H, m,  $CH_2$ -Gly),  
 3.73 (3H, s,  $OCH_3$ ), 3.25-3.02 (9H, overlapping m,  $2 \times CH_2 + 3$   
 $\times C_{\beta}H$ ), 2.84 (1H, m,  $C_{\beta}H$ ), 2.04 (1H, m,  $CH_2$ - $CH_2$ - $CH_2$ ), 1.71  
 (1H, m,  $C_{\gamma}H$ -Leu), 1.56 (2H, m,  $C_{\beta}H_2$ ), 1.36 (9H, s,  $OC(CH_3)_3$ ),  
 1.28 (9H, s,  $OC(CH_3)_3$ ), 0.94 (3H, d,  $J$  6.3, Leu- $CH_3$ ), 0.89  
 (3H, d,  $J$  6.3, Leu- $CH_3$ );  $\delta_c$  (90.5 MHz;  $CD_3OD$ ) 175.5, 175.1,  
 174.2 172.9, 172.6, 158.7, 158.2 ( $C=O$ ), 155.4, 138.3, 136.4,  
 133.6, 130.9, 130.2, 129.6, 128.0, 125.2, 121.2 (all ArC),  
 81.0, 79.6 ( $C_q$ ), 64.5, 58.0, 57.5, 54.4, 53.2, 47.6, 44.1,  
 41.0, 38.1, 38.0, 36.3, 30.8, 29.2, 28.7, 28.2, 25.9, 23.5,  
 21.7, 18.6, -1.5 ( $CH$ ,  $CH_2$  and  $CH_3$ );  $m/z$  ( $FAB^+$ ; NBA) 1064.5789  
 ( $[M+H]^+$ ,  $C_{53}H_{82}N_9O_{12}Si$  requires 1064.5852)

15



Scheme 5

**EXAMPLE 9**

Coupling of two functional groups to N<sup>f</sup> and N<sup>a</sup> (Scheme 6)

**2,6-Bis-tert-butoxycarbonylmethyl-7-methoxycarbonyl-5-oxo-5,6,7,8-tetrahydro-inimidazo[1,5-c]pyrimidin-2-ium;bromide**  
5 **(molecule 22)**

NaH 13 mg (0.307 mmol) was suspended in dried DMF (2 ml) at 0°C. A solution of compound 3 60 mg (0.307 mmol) in DMF (1 ml) was added slowly dropwise. The reaction mixture was stirred at 0°C for 30 min and at room temperature for 1h more  
10 until no gas was evolved. The reaction solution was cooled down to 0°C again. Bromoacetic acid tert-butyl ester 0.07 ml (0.461 mmol) was added by syringe very slowly at cold condition. The solution was stirred at 0°C for 30 min and at r.t. for 2hrs more. When the compound 3 was not detectable by  
15 TLC, bromoacetic acid tert-butyl ester 0.14 ml (0.921 mmol) was added once more dropwise. The reaction mixture was heated to 70°C for overnight. The reaction was monitored by TLC. After 15 hours of heating, the solution was cooled down to r.t. and concentrated in *vacuo*. The crude residue was treated  
20 with diethyl ether twice to remove excess of bromide and dried in *vacuo*. The crude compound 22 was used for next step without further purification. MS (ESI): m/z: 424.56 [M-Br]<sup>+</sup>

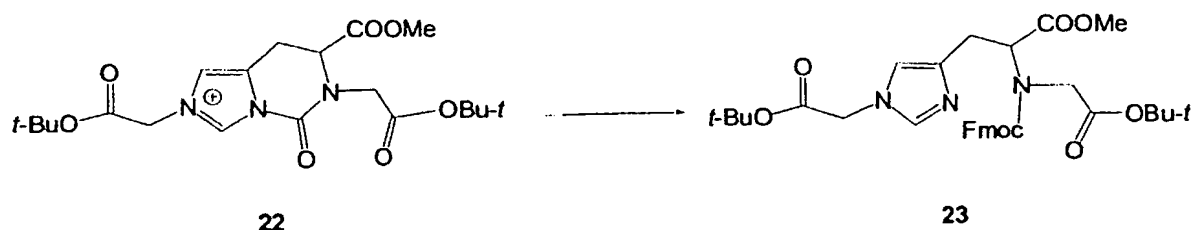
**2-[tert-Butoxycarbonylmethyl-(9H-fluoren-9-yl-methoxy**  
25 **carbonyl)-amino]-3-(1-tert-butoxycarbonylmethyl-1H-imidazol-4-yl)-propionic acid methyl ester (molecule 23)**

The crude compound 22 was dissolved in acetonitrile (10 ml) at r.t. Fm-OH 181 mg (0.921 mmol) and DIPEA 0.08 ml (0.461 mmol) were added. After 24 hours of stirring at r.t., the  
30 reaction solution was neutralized by adding 1N HCl solution and concentrated under reduced pressure. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and extracted with water once and 1N HCl



solution once. The organic layer was dried over  $\text{Na}_2\text{SO}_4$ , concentrated *in vacuo* and purified by flash column chromatography afforded compound 23 (yield: 40-50% from compound 3).

5



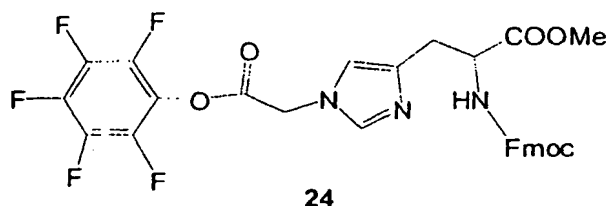
Scheme 6

10

**EXAMPLE 10**Preparation of activated histidine derivative

2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-(1-pentafluoro  
 15 phenyloxycarbonylmethyl-1H-imidazol-4-yl)propionic acid  
 methyl ester (molecule 24): Compound 9 (0.198 mmol) was  
 dissolved in THF (3 ml) and pyridine was added to neutralize  
 the solution pH 6-7. Pyridine 0.03 ml (0.396 mmol) was added.  
 Then after a solution of trifluoroacetic acid pentafluoro-  
 20 phenyl ester (TFA-Pfp) 111 mg (0.396 mmol) in THF (2 ml) was  
 added dropwise by syringe very slowly at r.t. After 19 hours  
 of stirring the reaction mixture at r.t., the solution was  
 concentrated *in vacuo*. The crude residue was dissolved in  
 dichloromethane and extracted with 0.5N HCl once, 0.5N  $\text{Na}_2\text{CO}_3$   
 25 once and brine once. The organic layer was dried over  $\text{Na}_2\text{SO}_4$ ,  
 concentrated *in vacuo*, and purified by flash column  
 chromatography afforded compound 24. (Yield: 55%); MS (ESI):  
 m/z: 615.78  $[\text{M}+\text{H}]^+$

33



24

**EXAMPLE 11****5 General coupling procedure of histidine derivative to peptide**

**Coupling:** Compound 9 or 24 was used for the coupling reactions. One of the ligands (nomally 0.02-0.08 mmol) was dissolved in DMF and Et<sub>3</sub>N or DIPEA (0.03-0.1 mmol) was added as a base. In case of compound 9, BOP or TBTU (normally

10 0.025-0.09 mmol) was added as a coupling reagent, so that the carboxyl acid group of the ligand was activated in 30 min at room temperature. Then after, a solution of a peptide (normally 0.01-0.02 mmol) in DMF, such as (beta)-hairpin loop peptide, RGD, or bombesin was added dropwise by syringe. In

15 case of compound 24, without coupling reagent, a peptide solution was added right after addition of base. The compound 24 is more suitable when the peptide has a free carboxylic acid group, such as Phe-Gly-OH, Gly-Pro-OH, Gastrin (7 free COOH in structure), or TOCA-OH. Depending on the peptide, the

20 reaction mixture was stirred 2-18 hours. The reaction was monitored by HPLC. When the peptide was not detectable by HPLC, the reaction solution was concentrated in vacuo. The crude residue was purified by preparative HPLC and the product was conformed by MS.

25

**Deprotection:** A histidine conjugated peptide (normally 0.003-0.006 mmol) was dissolved in piperidine (1 ml) at room temperature. After 30-40 min of stirring, the reaction mixture was poured into ice-cold water (3 ml). The white

solid, fulvine, was filtered and rinsed with water (1 ml). The aqueous solution was concentrated *in vacuo* to provide white solid as an all, Fmoc and methyl ester, deprotected product that was used for labeling without further  
5 purification.

**Labeling:** A solution of a conjugate ( $10^{-3}$  or  $10^{-4}$  M in water or phosphate buffer (pH7.4), 100  $\mu$ l) was added to a vial. Then a solution of  $[^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$  (900  $\mu$ l) was added to  
10 the vial (total concentration:  $10^{-4}$  or  $10^{-5}$  M). The solution was heated  $90^\circ\text{C}$  for 30 min to 1 hour. Normally the labeling was done quantitatively in 30 min in concentration of  $10^{-4}$  M and 20 to 50% in 30 min in concentration of  $10^{-5}$  M. In case of (beta)-hairpin loop peptide, its conjugate showed  
15 quantitative labeling in 30 min even in concentration of  $10^{-5}$  M.

## EXAMPLE 12

### General labeling procedures

20 A solution of ligand ( $10^{-3}$  or  $10^{-4}$  M in  $\text{H}_2\text{O}$ , 100  $\mu$ l) obtained from either organic synthesis or through rhenium oxidation pathway was added to a vial, which was then sealed and degassed with a stream of nitrogen gas for 10 min. A solution of  $[^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$  (900  $\mu$ l) was added to the vial  
25 via syringe and the vial was heated to  $70-90^\circ\text{C}$  for 30 min to yield the corresponding  $[^{99m}\text{Tc}(\text{CO})_3]^+$  complexes,  $[(5)^{99m}\text{Tc}(\text{CO})_3]$  and  $[(11)^{99m}\text{Tc}(\text{CO})_3]$  in high yield via HPLC with radioactive detection. All the results are described in Table 4.

Table 4

Method	Nr. of Ligand	Conc. (M)	Temp. (°C)	Time (min)	Yield (%)
Two step labeling	5	$10^{-4}$	70	30	quantitative
	5	$10^{-5}$	70	30	91 <sup>[a]</sup>
	5	$10^{-6}$	70	30	20 <sup>[b]</sup>
	11	$10^{-4}$	90	30	quantitative <sup>[c]</sup>
	11	$5 \times 10^{-5}$	90	30	quantitative
	11	$10^{-5}$	90	30	quantitative <sup>[d]</sup>
	11	$10^{-6}$	90	30	41 <sup>[e]</sup>
One pot labeling	11	$10^{-4}$	90	20	94
	11	$5 \times 10^{-5}$	90	20	96
	11	$10^{-5}$	90	20	34 <sup>[e]</sup>

[a]: The labeling was done quantitatively in 1h. Ligand 5 from the oxidation, at 75°C for 30 min yield was 85%.

5 [b]: The labeling reached 64% yield after 1.5h.

[c]: Ligand 11 from the oxidation showed 88% yield at 90°C for 30 min.

[d]: Ligand 11 from the oxidation showed 73% yield at 90°C for 30 min.

10 [e]: The labeling reached more than 65% yield after 1.5h.

## CLAIMS

1. Histidine derivatives, comprising a histidine that is any one of the following:

5 a) derivatized at the  $N^{\epsilon}$  and at least protected at the  $N^{\alpha}$  and optionally at the  $N^{\delta}$ ; or

b) derivatized at the  $N^{\alpha}$  and at least protected at the  $N^{\alpha}$  and optionally at the  $N^{\delta}$ ; or

c) derivatized at the  $N^{\epsilon}$  and  $N^{\alpha}$  and at least  
10 protected at the  $N^{\alpha}$  and optionally at the  $N^{\delta}$ ; or

d) derivatized at the  $N^{\epsilon}$ ; or

e) derivatized at the  $N^{\alpha}$ ; or

f) derivatized at the  $N^{\epsilon}$  and  $N^{\alpha}$ ; or

g) at least protected at the  $N^{\alpha}$  and optionally at the  
15  $N^{\delta}$ .

2. Histidine derivatives as claimed in claim 1, wherein the  $N^{\epsilon}$  and/or  $N^{\alpha}$  are derivatized with  $(CH_2)_n-R$  wherein  $n = 0-10$ , preferably 1-5, and R is a group selected from  $-NH_2$ ,  $-COOH$ ,  $-OH$ ,  $-X$  or  $-X'$ -biomolecule, wherein  $X'$  is a  
20 coupling block having a bond that is the result from a reaction between  $COOH$  and  $NH_2$ ,  $NH_2$  and  $COOH$ ,  $OH$  and  $Ph-OH$ , wherein Ph is phosphoric acid group on the biomolecules, such as phosphorylated peptide or glycosyl phosphates or X and an electrophilic functional group on the biomolecule, in  
25 particular S, OH or amine.

3. Histidine derivatives as claimed in claim 2, wherein X is selected from halides, azides, pseudohalides, phosphate, thiol, silyl.

4. Histidine derivatives as claimed in claim 1, wherein the  $N^{\epsilon}$  and/or  $N^{\alpha}$  are derivatized with a biomolecule.  
30

5. Histidine derivatives as claimed in claim 2,

wherein the biomolecule is selected from bombesine, (alpha)-MSH peptides, such as melanocortin, octreotate, somatostatin, interleukin-8 (IL8), CCK, (beta)-hairpin loop peptides, neurotensin, biotin, monoclonal antibodies, such as  
5 monoclonal antibodies directed against prostate membrane specific antigen (pmsa).

6. Histidine derivatives as claimed in claim 4, wherein the biomolecule is selected from bombesine, (alpha)-MSH peptides, such as melanocortin, octreotate, somatostatin,  
10 interleukin-8 (IL8), CCK, (beta)-hairpin loop peptides, neurotensin, biotin, monoclonal antibodies, such as monoclonal antibodies directed against prostate membrane specific antigen (pmsa).

7. Histidine derivatives as claimed in claim 1,  
15 wherein N<sup>α</sup> and N<sup>δ</sup> are protected with a carbonyl thus forming a six-membered urea ring.

8. Histidine derivatives as claimed in claim 1, wherein N<sup>α</sup>, N<sup>δ</sup> and carboxyl group are protected with a metal tricarbonyl.

20 9. Histidine derivatives as claimed in claim 1, wherein N<sup>α</sup> is protected with a amine protecting group, in particular Fmoc, Cbz, BOC, Teoc, methoxycarbonyl, or ethoxycarbonyl, and the carboxyl group is protected by esterification.

25 10. Histidine derivatives as claimed in claim 1, wherein the N<sup>α</sup>, N<sup>δ</sup> and carboxyl group are deprotected and instead coordinated to a radioactively labeled metal tricarbonyl.

30 11. Histidine derivatives as claimed in claim 2, wherein the N<sup>α</sup>, N<sup>δ</sup> and carboxyl group are deprotected and instead coordinated to a radioactively labeled metal tricarbonyl.

12. Histidine derivatives as claimed in claim 3, wherein the N<sup>α</sup>, N<sup>δ</sup> and carboxyl group are deprotected and instead coordinated to a radioactively labeled metal tricarbonyl.

5 13. Histidine derivatives as claimed in claim 10, wherein the radioactively labeled metal tricarbonyl is selected from [<sup>99m</sup>Tc(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>+</sup>, [<sup>188</sup>Re(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>+</sup> and [<sup>97</sup>Ru(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>2+</sup>

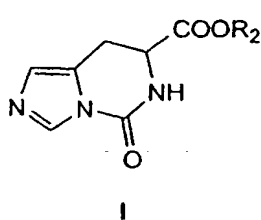
10 14. Histidine derivatives as claimed in claim 11, wherein the radioactively labeled metal tricarbonyl is selected from [<sup>99m</sup>Tc(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>+</sup>, [<sup>188</sup>Re(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>+</sup> and [<sup>97</sup>Ru(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>2+</sup>

15 15. Histidine derivatives as claimed in claim 12, wherein the radioactively labeled metal tricarbonyl is selected from [<sup>99m</sup>Tc(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>+</sup>, [<sup>188</sup>Re(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>+</sup> and [<sup>97</sup>Ru(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>2+</sup>

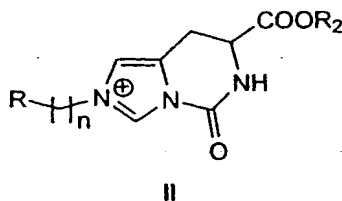
16. Histidine derivatives as claimed in claim 1, (instead of claim 1, claim 2 cannot be more proper?) wherein the N<sup>ε</sup> is derivatized with -(CH<sub>2</sub>)<sub>n</sub>-COO-pentafluorophenyl.

20 17. Histidine derivative as claimed in claim 16, wherein the N<sup>α</sup> and the carboxyl are protected.

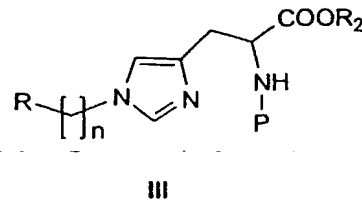
18. Histidine derivative having one of the following structural formulas:



R<sub>2</sub> = H, methyl

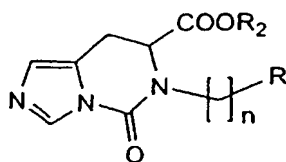


R = NH<sub>2</sub>, COOR<sub>1</sub>, OH, X,  
X'-biomolecule  
R<sub>1</sub> = H, *t*-butyl  
R<sub>2</sub> = H, methyl



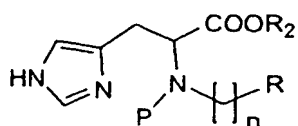
R = NH<sub>2</sub>, COOR<sub>1</sub>, OH, X, X'-biomolecule  
R<sub>1</sub> = H, *t*-butyl, Pfp  
R<sub>2</sub> = H, methyl  
P = H, Fmoc, Cbz, BOC, Teoc,  
methoxycarbonyl, ethoxycarbonyl

39



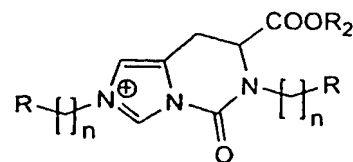
IV

R = H, COOR<sub>1</sub>  
 R<sub>1</sub> = H, *t*-butyl  
 R<sub>2</sub> = H, methyl



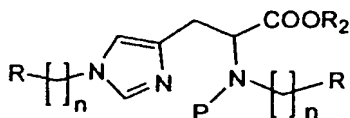
V

R = H, COOR<sub>1</sub>  
 R<sub>1</sub> = H, *t*-butyl  
 R<sub>2</sub> = H, methyl  
 P = H, Fmoc



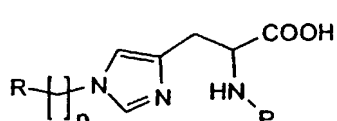
VI

R = NH<sub>2</sub>, COOR<sub>1</sub>, OH, X  
 R<sub>1</sub> = H, *t*-butyl  
 R<sub>2</sub> = H, methyl



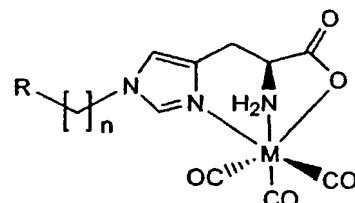
VII

R = H, COOR<sub>1</sub>  
 R<sub>1</sub> = H, *t*-butyl  
 R<sub>2</sub> = H, methyl  
 P = H, Fmoc



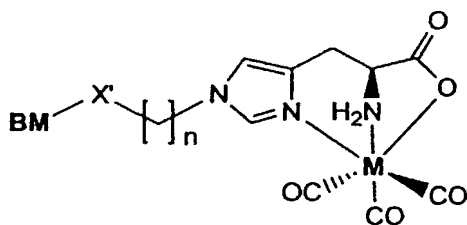
VIII

R = H, COOR<sub>1</sub>  
 R<sub>1</sub> = H, *t*-butyl  
 P = H, Fmoc



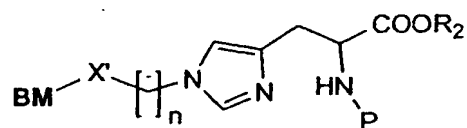
IX

M = Re, <sup>99m</sup>Tc, Ru  
 R = NH<sub>2</sub>, COOH, OH, X



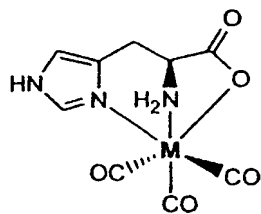
X

M = Re, <sup>99m</sup>Tc, Ru  
 BM = biomolecule



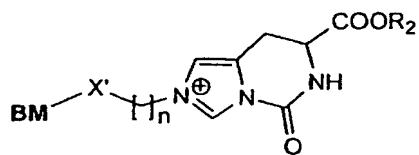
XI

R<sub>2</sub> = H, methyl  
 P = H, Fmoc, Cbz, Teoc



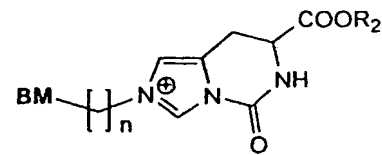
XII

M = Re, <sup>99m</sup>Tc



XIII

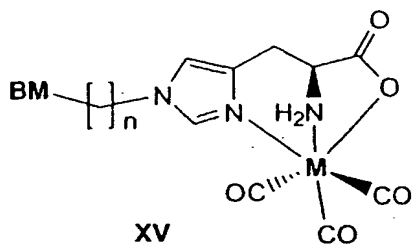
R<sub>2</sub> = H, methyl  
 BM = biomolecule



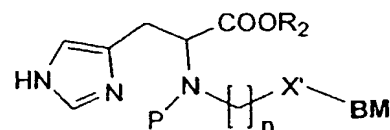
XIV

R<sub>2</sub> = H, methyl  
 BM = biomolecule

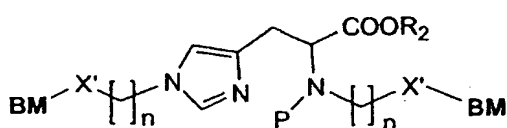




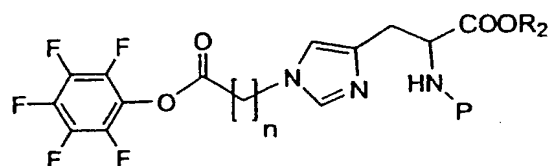
M = Re, <sup>99m</sup>Tc  
BM = biomolecule



R<sub>2</sub> = H, methyl  
P = H, Fmoc  
BM = biomolecule



R<sub>2</sub> = H, methyl  
P = H, Fmoc  
BM = biomolecule



R<sub>2</sub> = H, methyl  
P = H, Fmoc

5

19. Biomolecule coupled to a histidine derivative as claimed in claim 1.

20. Biomolecule as claimed in claim 19, wherein the biomolecule is selected from bombesine, (alpha)-MSH peptides, such as melanocortin, octreotate, somatostatin, interleukin-8 (IL8), CCK, (beta)-hairpin loop peptides, neurotensin, biotin, monoclonal antibodies, such as monoclonal antibodies directed against prostate membrane specific antigen (pmsa).

21. Biomolecule as claimed in claim 19, which biomolecule is labeled with a radioactively labeled metal tricarbonyl.

22. Method as claimed in claim 21, wherein the radioactively labeled metal tricarbonyl is selected from [<sup>99m</sup>Tc(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>+</sup>, [<sup>188</sup>Re(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>+</sup> and [<sup>97</sup>Ru(OH<sub>2</sub>)<sub>3</sub>(CO)<sub>3</sub>]<sup>2+</sup>.

23. Method for preparing histidine derivatives as claimed in claim 1, comprising:

- a) providing histidine;
- b) protecting at least the N<sup>α</sup> and optionally the carboxyl and the N<sup>δ</sup>;
- c) derivatizing at least one of the N<sup>ε</sup> and N<sup>α</sup>; and
- 5 d) deprotecting the protected groups.

24. Method as claimed in claim 23 further comprise the step e) of labeling the deprotected compound to obtain a labelled compound.

25. Method as claimed in claim 23, wherein the N<sup>α</sup> and  
10 N<sup>δ</sup> are protected by a carbonyl group thus forming a six-membered urea ring.

26. Method as claimed in claim 23, wherein the carboxyl, N<sup>α</sup> and N<sup>δ</sup> are coordinated to a metal, in particular a metal tricarbonyl.

- 15 27. Method as claimed in claim 23, wherein the carboxyl is protected by esterification and the N<sup>α</sup> is protected with an amine protecting group, in particular Fmoc, Cbz, BOC, Teoc, methoxycarbonyl, or ethoxycarbonyl.

28. Method as claimed in claim 23, wherein the N<sup>ε</sup>  
20 and/or N<sup>α</sup> are derivatized with (CH<sub>2</sub>)<sub>n</sub>-R wherein n = 0-10, preferably 1-5, and R is a group selected from -NH<sub>2</sub>, -COOH, -OH, -X or -X'-biomolecule, wherein X' is a coupling block having a bond that is the result from a reaction between COOH and NH<sub>2</sub>, NH<sub>2</sub> and COOH, OH and Ph-OH,  
25 wherein Ph is phosphoric acid group on the biomolecules, such as phosphorylated peptide or glycosyl phosphates or X and an electrophilic functional group on the biomolecule, in particular S, OH or amine.

29. Method as claimed in claim 23, wherein the N<sup>ε</sup>  
30 and/or N<sup>α</sup> are derivatized with a biomolecule.

30. Method as claimed in claim 23, wherein the N<sup>ε</sup> is

derivatized with  $-(\text{CH}_2)_n\text{-COO-pentafluorophenyl}$ .

31. Method as claimed in claim 24, wherein the compound is labeled with a radioactively labeled metal tricarbonyl.

- 5        32. Method as claimed in claim 31, wherein the radioactively labeled metal tricarbonyl is selected from  $[\text{}^{99\text{m}}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ ,  $[\text{}^{188}\text{Re}(\text{OH}_2)_3(\text{CO})_3]^+$  and  $[\text{}^{97}\text{Ru}(\text{OH}_2)_3(\text{CO})_3]^{2+}$

Figure 1.

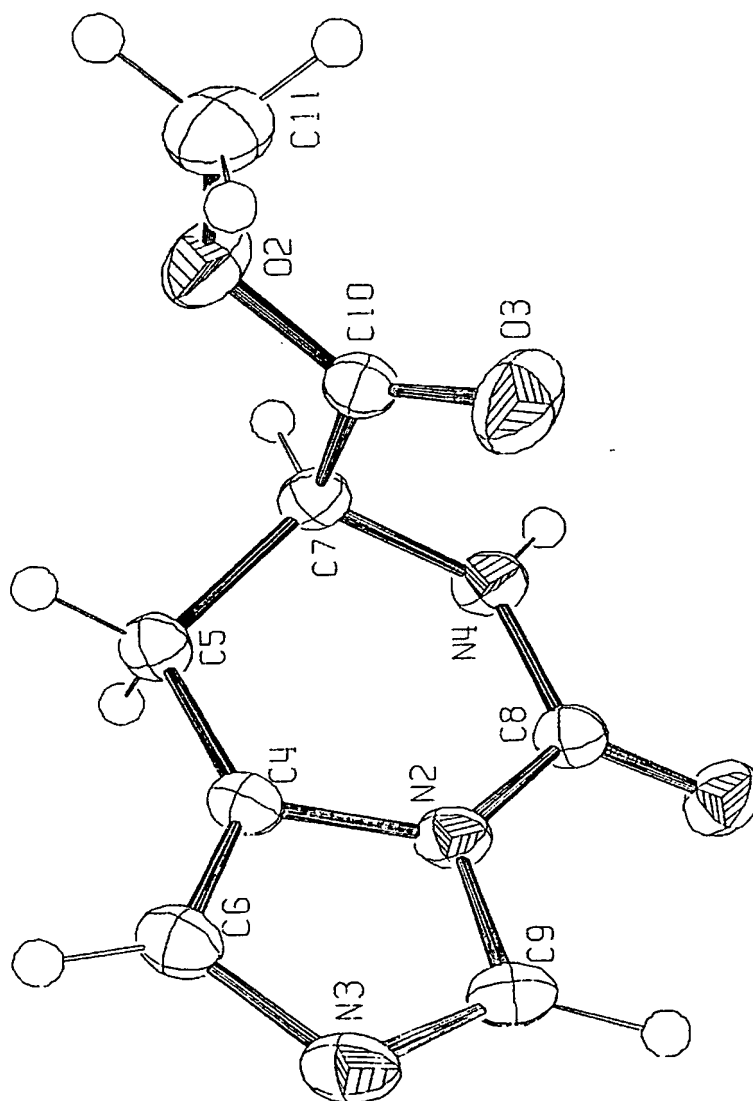


Figure 2

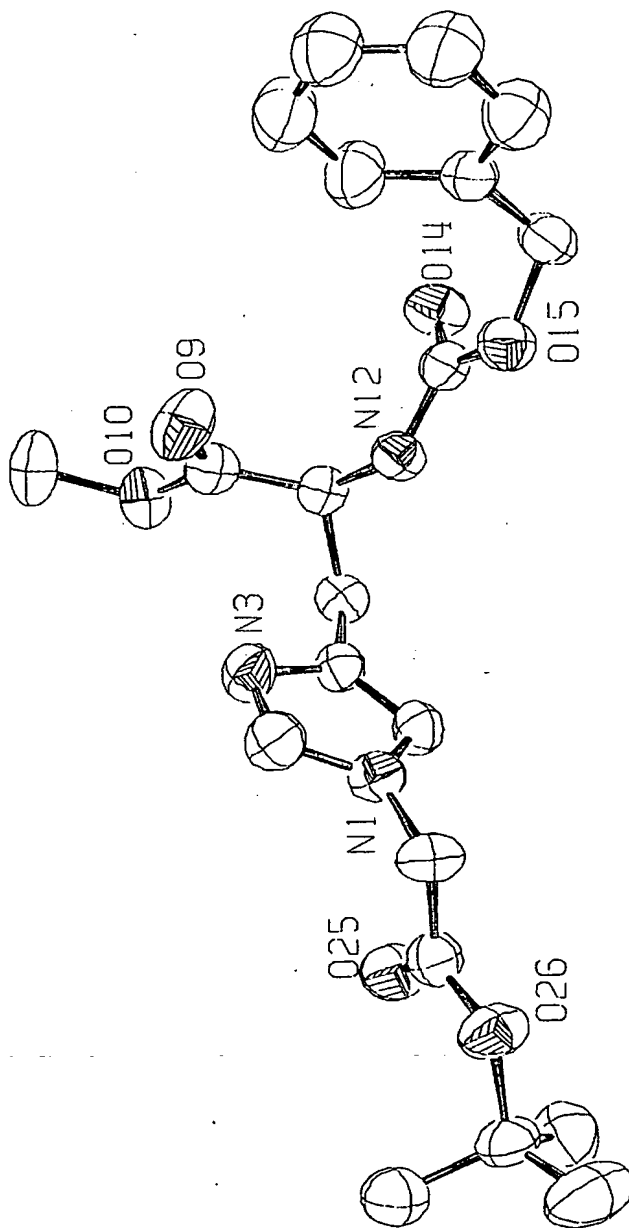
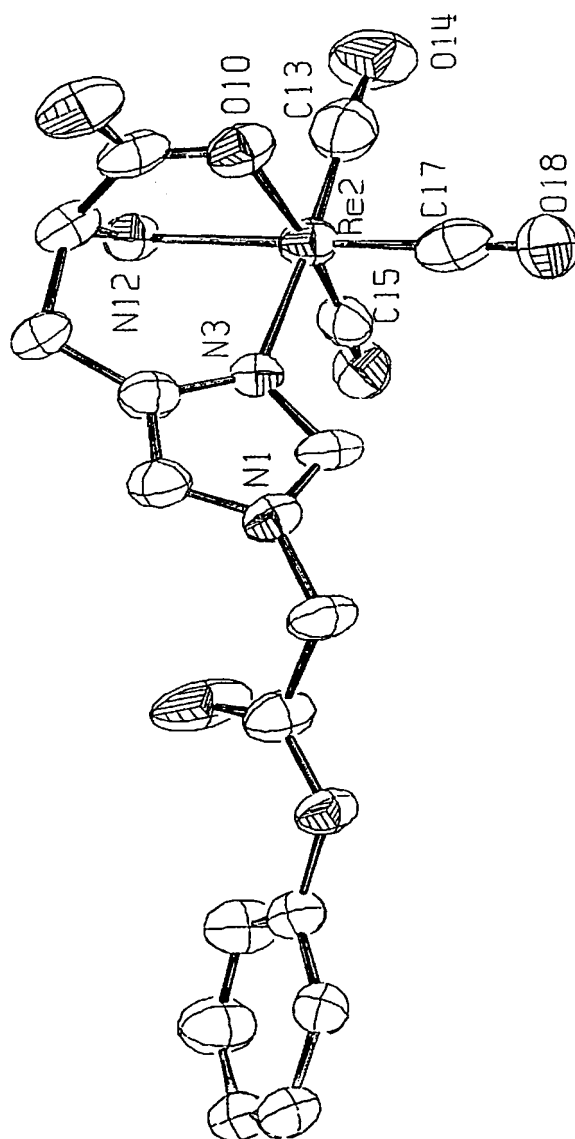


Figure 3



(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
11 November 2004 (11.11.2004)

PCT

(10) International Publication Number  
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(25) Filing Language: English

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(30) Priority Data:  
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(71) Applicant (for all designated States except US): **UNIVERSITÄT ZÜRICH** [CH/CH]; Rämistrasse 71, CH-8006 Zürich (CH).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **ALBERTO, Roger** [CH/CH]; St. Georgenstrasse 54, CH-8400 Winterthur (CH). **PAK, Jaw, Kyoung** [KR/CH]; Raingaeßli, CH-6340 Baar-Inwil (CH).

(74) Agent: **VAN SOMEREN, Petronella, Francisca, Hendrika, Maria**; Arnold & Siedsma, Sweelinckplein 1, NL-2517 GK The Hague (NL).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AI, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

(88) Date of publication of the international search report:  
19 May 2005

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: N<sup>ε</sup> AND/OR N<sup>α</sup> DERIVATIZED, METAL AND ORGANIC PROTECTED L-HISTIDINE FOR COUPLING TO BIOMOLECULES FOR HIGHLY EFFICIENT LABELING WITH [M(OH)<sub>2</sub>(CO)<sub>3</sub>]<sup>+</sup> BY FAC COORDINATION

(57) Abstract: The present invention relates to novel histidine derivatives that can be used for the labeling of biomolecules with radioactive metal tricarbonyls. The new derivatives have a histidine that is derivatized at the N<sup>ε</sup> and at least protected at the N<sup>α</sup> and optionally at the N<sup>δ</sup>; or derivatized at the N<sup>α</sup> and at least protected at the N<sup>ε</sup> and optionally at the N<sup>δ</sup>; or derivatized at the N<sup>ε</sup> and at least protected at the N<sup>α</sup> and optionally at the N<sup>δ</sup>; or derivatized at the N<sup>ε</sup>; or derivatized at the N<sup>α</sup>; or derivatized at the N<sup>δ</sup> and N<sup>α</sup>; or at least protected at the N<sup>α</sup> and optionally at the N<sup>δ</sup>.

WO 2004/097406 A3

## INTERNATIONAL SEARCH REPORT

PCT/EP2004/004683

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 7 G01N33/534 A61K51/08

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 7 G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, CHEM ABS Data, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 571 792 A (BOLTON GARY L ET AL) 5 November 1996 (1996-11-05) column 9	1, 2, 18
X	WO 92/00068 A (SMITHKLINE BEECHAM CORP) 9 January 1992 (1992-01-09) page 6	1
X	WO 97/26246 A1 (WARNER-LAMBERT COMPANY; DOHERTY, ANNETTE, MARIAN; KALTENBRONN, JAMES,) 24 July 1997 (1997-07-24) page 103 - page 109	1
X	US 5 824 803 A (CONRAD DAVID W ET AL) 20 October 1998 (1998-10-20) column 3	1
	----- --/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

26 January 2005

Date of mailing of the international search report

25.02.05

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3016

Authorized officer

Diez Schlereth, D



## INTERNATIONAL SEARCH REPORT

PCT/EP2004/004683

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BELLA LA R ET AL: "A '99mTc(I)-postlabelled high affinity bombesin analogue as a potential tumor imaging agent" BIOCONJUGATE CHEMISTRY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, US, vol. 13, no. 3, 15 May 2002 (2002-05-15), pages 599-604, XP002218739 ISSN: 1043-1802 page 600; figure 1	1,4,6,8, 10, 18-24, 29,31,32
X	BULLOK KRISTIN E ET AL: "Characterization of novel histidine-tagged Tat-peptide complexes dual-labeled with 99mTc-tricarboxyl and fluorescein for scintigraphy and fluorescence microscopy." BIOCONJUGATE CHEMISTRY, vol. 13, no. 6, 2002, - 18 October 2002 (2002-10-18) pages 1226-1237, XP002315204 ISSN: 1043-1802 figure 1	1,8,10, 13,19, 21-23,26
A	WO 01/00637 A (ALBERTO ROGER ; DYSZLEWSKI MARY M (US); BUGAJ JOSEPH E (US); MALLINCKR) 4 January 2001 (2001-01-04) the whole document	1-32
A	WO 98/48848 A (ALBERTO ROGER ; SCHIBLI ROGER (CH); EGLI ANDRE (CH); MALLINCKRODT MEDI) 5 November 1998 (1998-11-05) the whole document	1-32
A	ALBERTO R ET AL: "SYNTHESIS AND PROPERTIES OF BORANOCARBONATE: A CONVENIENT IN SITU CO SOURCE FOR THE AQUEOUS PREPARATION OF A99MTC(OH2)3(CO)3U+ " JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC, US, vol. 123, 13 March 2001 (2001-03-13), pages 3135-3136, XP001120003 ISSN: 0002-7863 cited in the application the whole document	1-32
A	DU J ET AL: "Technetium-99m labelling of glycosylated somatostatin-14" APPLIED RADIATION AND ISOTOPES, PERGAMON PRESS LTD., EXETER, GB, vol. 55, no. 2, August 2001 (2001-08), pages 181-187, XP004231602 ISSN: 0969-8043 the whole document	1-32

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## INTERNATIONAL SEARCH REPORT

PCT/EP2004/004683

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHIBLI R ET AL: "Influence of the denticity of ligand systems on the in vitro and in vivo behaviour of <sup>99m</sup> Tc(I)-tricarbonyl complexes: a hint for the future functionalisation of biomolecules" BIOCONJUGATE CHEMISTRY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, US, vol. 11, no. 3, May 2000 (2000-05), pages 345-351, XP002218742 ISSN: 1043-1802. ----- figure 1	18
X	SCHIBLI ROGER ET AL: "Current use and future potential of organometallic radiopharmaceuticals." EUROPEAN JOURNAL OF NUCLEAR MEDICINE AND MOLECULAR IMAGING. NOV 2002, vol. 29, no. 11, November 2002 (2002-11), pages 1529-1542, XP002315205 ISSN: 1619-7070 figure 5 -----	18
P,X	J.K. PAK ET AL: "N-epsilon functionalization of metal and organic protected L-histidine for a highly efficient, direct labeling of biomolecules with <sup>99m</sup> Tc(OH <sub>2</sub> ) <sub>3</sub> (CO) <sub>3</sub> !" CHEMISTRY EUROPEAN JOURNAL, vol. 9, 2003, pages 2053-2061, XP002303582 the whole document -----	1-32

## INTERNATIONAL SEARCH REPORT

PCT/EP2004/004683

**Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1-32 (partially)  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-32 (partially)

histidine derivatives derivatized at the alpha and at the epsilon nitrogens, biomolecules coupled thereto and methods for their preparation (claims 1-32, partially, as concerning options a, c and f of claim 1 and formulae II, VI, IX-X and XIII-XV of claim 18)

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2. claims: 1-32 (partially)

histidine derivatives derivatized at the epsilon nitrogen, biomolecules coupled thereto and methods for their preparation (claims 1-32, partially, as concerning option d of claim 1 and formulae II, III, VI-XI, XIII-XV and XVII-XVIII of claim 18)

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3. claims: claims 1-32 (partially)

histidine derivatives derivatized at the alpha nitrogen, biomolecule coupled thereto and method for their preparation (claims 1-32, partially, as concerning options e and g of claim 1 and formulae I-II, IV, VI, IX-X and XII-XV of claim 18).

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1-32 (partially)

The scope of present claim 1 embraces an infinite number of compounds and only few part of them is supported by the text of the present application. Therefore, the claim does not meet the requirements of Art. 6 PCT in combination with Art. 5 PCT. For this reason, it is not possible to carry out a complete meaningful search embracing the whole scope of this claim. Analogous arguments apply for the subject-matter of claim 18. Thus, the present search report has to be considered as being incomplete in the sense that only a few and limited number of examples embraced within the scope of the Markush-type definitions/formulae of independent claims 1 and 18 could be searched (for further information, see attached WO-ISA).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

## INTERNATIONAL SEARCH REPORT

PCT/EP2004/004683

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 5571792	A	05-11-1996	AT 200085 T	15-04-2001
			AU 699629 B2	10-12-1998
			AU 2650495 A	25-01-1996
			CA 2190977 A1	11-01-1996
			DE 69520499 D1	03-05-2001
			DE 69520499 T2	26-07-2001
			DK 767800 T3	18-06-2001
			EP 0767800 A1	16-04-1997
			ES 2158115 T3	01-09-2001
			GR 3035936 T3	31-08-2001
			HR 950337 A1	31-08-1997
			JP 10502078 T	24-02-1998
			NO 965604 A	25-02-1997
			NZ 287696 A	19-12-1997
			PT 767800 T	30-08-2001
			SI 767800 T1	31-08-2001
			WO 9600736 A1	11-01-1996
			ZA 9505411 A	05-02-1996
WO 9200068	A	09-01-1992	AU 650890 B2	07-07-1994
			AU 8105991 A	23-01-1992
			CA 2084776 A1	29-12-1991
			EP 0536262 A1	14-04-1993
			IE 912248 A1	01-01-1992
			JP 5508642 T	02-12-1993
			MX 9100018 A1	03-02-1992
			NZ 238688 A	26-05-1992
			PT 98143 A	30-04-1992
			WO 9200068 A1	09-01-1992
			US 5565480 A	15-10-1996
			US 5444081 A	22-08-1995
WO 9726246	A1	24-07-1997	ZA 9104960 A	29-07-1992
			AU 1529997 A	11-08-1997
			US 6008372 A	28-12-1999
US 5824803	A	20-10-1998	ZA 9700327 A	22-07-1997
			US 5922552 A	13-07-1999
WO 0100637	A	04-01-2001	US 6194184 B1	27-02-2001
			AT 277939 T	15-10-2004
			AU 6488900 A	31-01-2001
			CA 2377018 A1	04-01-2001
			CZ 20014603 A3	13-11-2002
			DE 60014375 D1	04-11-2004
			EP 1192166 A1	03-04-2002
			HU 0301779 A2	28-08-2003
			JP 2003503415 T	28-01-2003
			WO 0100637 A1	04-01-2001
WO 9848848	A	05-11-1998	EP 0879606 A1	25-11-1998
			AT 211924 T	15-02-2002
			AU 748213 B2	30-05-2002
			AU 7141398 A	24-11-1998
			BR 9809409 A	13-06-2000
			CA 2282563 A1	05-11-1998
			DE 69803205 D1	21-02-2002
			DE 69803205 T2	19-09-2002

## INTERNATIONAL SEARCH REPORT

PCT/EP2004/004683

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9848848	A	DK 1019095 T3	06-05-2002
		EP 1019095 A1	19-07-2000
		ES 2168751 T3	16-06-2002
		HU 0003255 A2	28-02-2001
		JP 2002512616 T	23-04-2002
		NO 995160 A	13-12-1999
		NZ 337303 A	22-12-2000
		PL 336382 A1	19-06-2000
		PT 1019095 T	31-07-2002
		WO 9848848 A1	05-11-1998
		US 6344178 B1	05-02-2002

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